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Effect of Hypoxia on Levels of RNA Degradation Proteins in Mycobacterium smegmatis

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Effect of Hypoxia on Levels of RNA Degradation Proteins in *Mycobacterium smegmatis*

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Abstract

Mycobacterium tuberculosis, the bacterium that causes tuberculosis, can enter non-growing states in which it is phenotypically tolerant of antibiotics. These states are characterized by reduced metabolic activity, and occur in response to stressors encountered in the body, such as hypoxia and starvation. Both *M. tuberculosis* and its non-pathogenic relative *M. smegmatis* have been shown to stabilize their mRNA transcripts under growth-inhibiting conditions. One explanation for this phenomenon could be a decrease in the intracellular levels of mRNA degradation proteins. In the present research, several proteins fitting that description were epitope-tagged and their abundance measured under stress. Our results suggest that RNase E may be specifically downregulated in hypoxia.

Introduction

Though great strides have been made in recent decades, tuberculosis continues to pose a threat worldwide. The World Health Organization estimates that in 2014, 9.6 million people were infected and 1.5 million died of the disease (WHO, 2015). One of the major obstacles to treating tuberculosis is the bacteria's tendency toward a state of near-dormancy described as quiescence, which has been observed for some time in the laboratory (Wayne, 1976) and is distinguished from true dormancy by the continued presence of (a reduced amount of) ATP (Gengenbacher et al., 2010), low-level respiratory functions, and maintenance of membrane potential (Rao et al., 2008). A quiescent state can lead to a transient, non-heritable form of antibiotic resistance termed antibiotic tolerance (Levin & Rozen, 2006). There are several proposed mechanisms for this phenomenon, but a particularly compelling explanation is based on the decrease in bacterial metabolic activity. As early as 1944, researchers had begun to explore this explanation, with a finding that penicillin only killed *Staphylococcus aureus* in its actively growing state (Lee, Foley, & Epstein, 1944). More recently, this effect has been observed in mycobacteria, with a finding that *Mycobacterium tuberculosis* shows reduced killing from certain antibiotics after six weeks of starvation (Betts et al., 2002). Some researchers have also begun to explore the possibility that active remodeling of metabolic pathways also contributes to this antibiotic tolerance (Nguyen et al., 2011).

Due in part to the bacterium's ability to enter this quiescent state, treatment for tuberculosis is only successful when patients adhere to a lengthy course of antibiotics (Thomas et al., 2005). Noncompliance occurs at high rates, particularly among vulnerable populations and in patients who lack easy access to a clinic (Brudney & Dobkin, 1991; Shargi & Lindtjørn, 2007). Additionally, temporary physiological antibiotic tolerance in protected sub-populations of bacteria can lead to greater rates of genetic antibiotic resistance (Levin & Rozen, 2006). The granulomas that occur in tuberculosis are thought to induce this state, and typical research parameters to mimic those conditions include hypoxia (Wayne & Hayes, 2003) and nutrient starvation (Betts et al., 2002).

These problems point to a need for the development of faster-working tuberculosis treatments, particularly treatments capable of interfering with the quiescent state. In order to

achieve this, it is necessary to conduct significant further research into the mechanisms that allow bacteria to enter this non-growing state.

Research in a variety of bacterial species has suggested that mRNA transcript half-lives are increased when growth is arrested; this is referred to as mRNA stabilization. In *Bacillus subtilis,* transcripts expressed in the stationary phase had half-lives as much as 15 times longer than transcripts expressed during the exponential phase (Resnekov et al., 1990). In *E. coli,* the mRNA encoding a ribosome modulation factor was found to be stabilized during stationary phase (Aiso et al., 2005), and other research determined that anaerobic conditions caused slowing of mRNA turnover (Georgellis et al., 1993). Increased mRNA half-life during nutrient starvation has been observed in *Lactococcus lactis* (Redon et al. 2005, Dressaire et al. 2013), *Rhizobium leguminosarum* (Thorne & Williams 1997), and *Vibrio* sp. S14 (Nyström et al. 1990). In *M. tuberculosis,* hypoxic conditions induce mRNA stabilization (Rustad et al., 2012). Preliminary results from the Shell lab show similar results in *M. smegmatis,* a fast-growing non-pathogenic relative of *M. tuberculosis,* under conditions of hypoxia and carbon starvation. mRNA stabilization may be important for conserving energy in the presence of limited resources.

Beginning with *E. coli,* researchers have identified bacterial protein complexes, referred to as degradosomes, that are major participants in the process of mRNA degradation. Two major components of the degradosome in *E. coli* are RNase E, an essential endonuclease, and polynucleotide phosphorylase (PNPase) (Miczak et al., 1996). The propensity of bacterial degradation proteins to function in complexes seems to be conserved across many bacterial classes, with a similar complex having been identified in *S. aureus* (Roux et al., 2011) and a more transiently-associated complex characterized in *B. subtilis* (Cascante-Estepa et al., 2016; Lehnik-Habrink et al., 2010). Homologs of some of the *E. coli* degradosome component proteins exist in mycobacteria, including RNase E and PNPase (Kovacs et al., 2005, Unciuleac & Shuman, 2013). RNase J has also been identified as a protein involved in RNA degradation in Mycobacteria (Taverniti et al. 2011). However, it is unknown if these proteins function in complexes.

In the published literature that details transcriptome-wide analyses of *M. tuberculosis* under stress, there has been no identification of any alteration in the abundance of mRNA transcripts encoding RNases. However, under conditions of both hypoxia and nutrient starvation, several genes predicted to encode RNA helicases have been shown to down-regulated (Betts et al., 2002; Sherman et al, 2001). Helicases are known to be involved in RNA degradation and are constituents of the *E. coli* degradosome (reviewed in Houseley & Tollervey, 2009).

Recent research from Schubert et al. (2015) has yielded data regarding protein abundance in *M. tuberculosis* throughout hypoxic growth. The proteome-wide analysis does not show any significant difference in RNase protein levels in hypoxic growth as compared to log phase; however, the RNA helicase RhlE had decreased abundance in hypoxia, consistent with previously published transcriptional data (Betts et al., 2002).

The present research investigates whether levels of RNA degradation proteins are altered under hypoxic conditions in mycobacteria, in order to determine whether the levels of these proteins could be a factor in mRNA stabilization. Using *M. smegmatis,* several proteins associated

with RNA degradation (including RNase E, RNase J, and a putative RNA helicase) were epitope-tagged for quantification via western blot under conditions associated with quiescence.

Materials & Methods

Incubation

All liquid cultures were incubated at 37° C in a Lab-Line® incubator-shaker set to 175 rpm. *M. smegmatis* was grown in 7H9 supplemented with glycerol, 0.05% Tween 80, Bovine Serum Albumin Fraction V, NaCl, and catalase. *E. coli* was grown in LB broth. All plates were incubated in a dedicated warm room kept at 37° C. *M. smegmatis* was grown on 7H10 plates and *E. coli* was grown on LB agar plates.

Oligos

Primers and oligos were synthesized by IDT and resuspended at 100 µM in Qiagen elution buffer. Further dilutions were made with ultra-pure water.

PCR

PCR was done in 25 µl reactions with 2.5µl 10x NEB Taq buffer, 0.125 µl NEB Taq polymerase, 0.5 µl of each primer (10 µM), 0.5 µl of dNTPs and 1 µl of template DNA. Primers are listed in table 2.

E. coli Transformation

Transformation of *E. coli* was done with 12 µl of NEB 5-alpha competent cells and 1 µl of plasmid. Cells were incubated for 30 minutes on ice, heat-shocked for 30 seconds in a 42° C water bath, and then incubated for 5 more minutes on ice. 200 µl of SOC media was added, and then tubes were placed in the 37° C warm room and rotated for 1.5 hours. 150 µl and 20 µl of the transformation was plated on two plates.

M. smegmatis Electrocompetent Cells

Unless otherwise specified, *M. smegmatis* electrocompetent cells were made by washing overnight cultures of mc²155 with 10% glycerol. Cultures were grown from glycerol stocks overnight in 7H9. The cultures were pelleted at 3900 rpm, the supernatant was discarded, and the pellet was resuspended in a volume of 10% glycerol equivalent to the volume of the initial culture. This is done twice. The process is then repeated, but the cells are resuspended in 10% of initial volume, and then finally 1% of initial volume.

M. smegmatis Transformation

Basic (non-recombineering) transformation of *M. smegmatis* was done using 30-50 µl of competent cells and around 200 ng of plasmid, which were transferred to an electroporation

cuvette and electroporated at 2.5 kV with a Bio-Rad MicroPulser™. The mixture was then transferred into new 1.5 ml Eppendorf tubes and rotated for 2-4 hours at 37° C, then plated on 7H10 plates with appropriate antibiotic concentration (see table 1).

Table 1: Concentrations of antibiotic used in liquid culture

	E. coli	***M. smegmatis***
Kanamycin	50 μg/ml	20 μg/ml
Chloramphenicol	34 μg/ml	not used
Hygromycin	150 μg/ml	150 μg/ml

Recombineering

The recombineering system used was the ORBIT system, developed by Kenan Murphy at the University of Massachusetts Medical School, and plasmids were obtained from his lab. Plasmids used were pKM444 and pKM461 (plasmids for facilitating recombineering expressing RecT and Bxb1 integrase; kanamycin resistant) and pKM446 (plasmid containing epitope tag; hygromycin and chloramphenicol resistant). pKM461 differs from pKM444 by presence of sacR and sacB, counterselectable markers for sucrose.

Production of more plasmid was accomplished through transformation of 1 μl plasmid into 12 μl NEB 5-alpha competent cells, following above protocol. Plasmid extraction was done using either the Qiagen or Zymo Research miniprep kit, following the respective manufacturers' instructions.

Epitope tagging the proteins was accomplished through recombineering based on protocols in Murphy et al. 2015 and advisory from the Murphy lab. 5 ml cultures of strains of mc^2155 transformed with pKM461 were grown up overnight, then scaled up to a culture with 5 ml per transformation + an additional 5 ml. At an OD of 0.5, cultures were induced with 500 ng/ml ATc and placed back in the shaker for an additional 3 hours. The cells were then made electrocompetent as described above, without the final step of resuspending in 1% of the original volume, so the cells remained at 10% of their original volume. Additionally, tubes remained on ice during the competent cell procedure and spins were done at 4° C. 400 μl of the competent cells were used for each transformation, which was done with 400 ng of pKM446 and 1 μg of recombineering oligo (see below for description; sequences in table 2). An additional control transformation contained plasmid but no oligo. The transformations were done at 2.5 kV, 1000 Ω, and 25 μF. The results were transferred to a tube containing 2 ml 7H9 and rotated overnight (12-16 hours) and then plated on 7H10 plates with hygromycin.

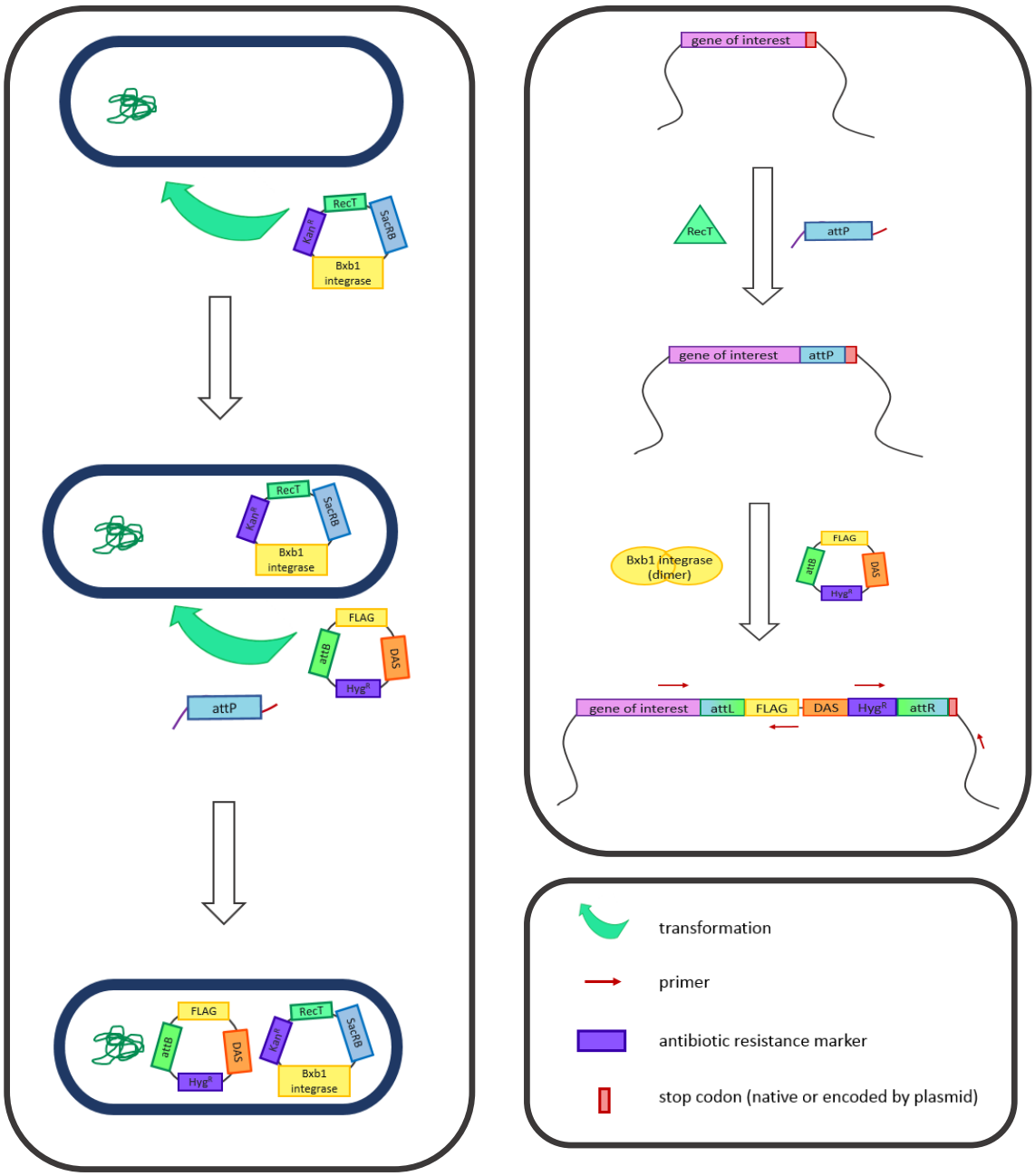

Figure 1: Schematic of the ORBIT recombineering system. First, the recombineering plasmid (encoding RecT and Bxb1 integrase, which facilitate incorporation of DNA into the genome, and the counterselectable marker SacRB) is transformed into the lab strain of *M. smegmatis*. Then a second transformation includes the oligo containing the attP site and homology to the target site in the chromosome and the plasmid containing the FLAG tag. RecT facilitates the integration of the oligo into the bacterial genome (detailed diagram of this process in Figure 2). The Bxb1 integrase then facilitates the site-specific recombination at the attP site, and the full plasmid is incorporated into the genome. The attL and attR sites on either side of the plasmid DNA are each formed from half the attP and half the attB site. Sites of PCR primers for junction verification are shown in red.

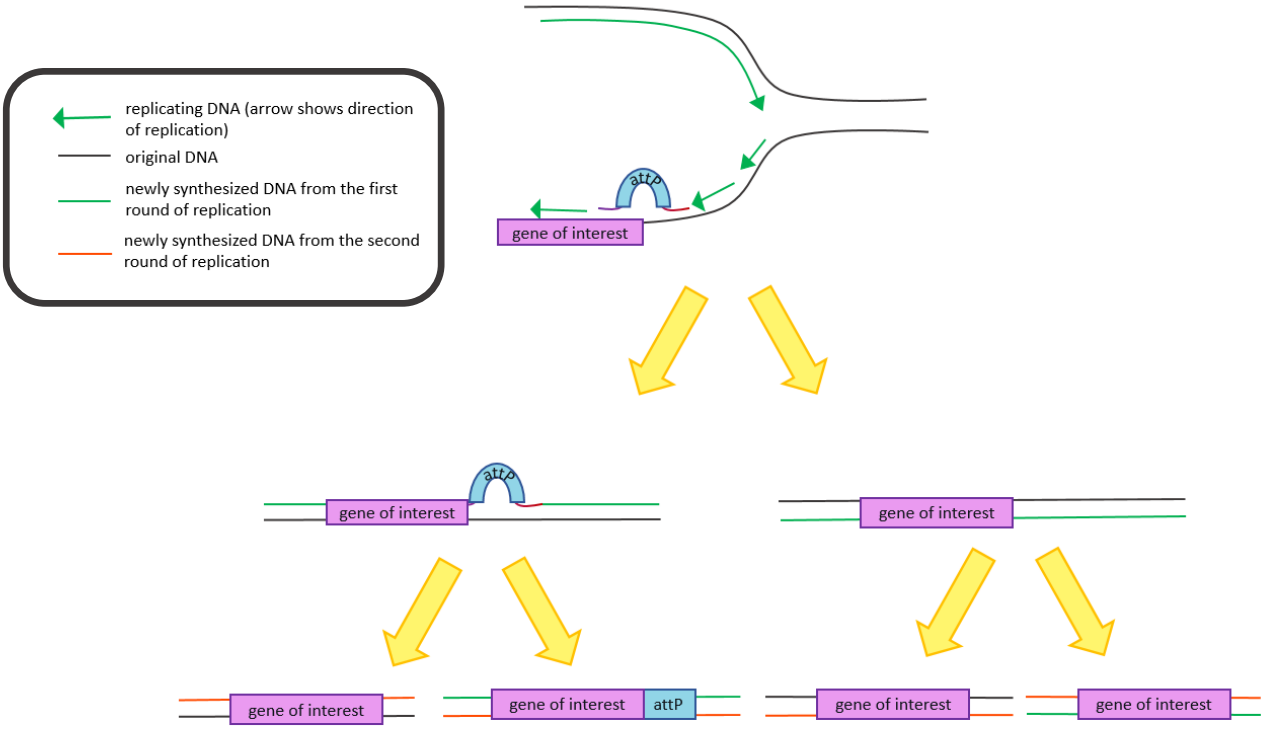

Figure 2: Depiction of the incorporation of oligo in the ORBIT system. The oligo is treated as an Okazaki fragment and incorporated as part of the lagging strand during normal genome replication. The excess DNA corresponding to the attP site exists as a loop of unpaired DNA until the second round of replication, at which point one of the daughter cells will contain a double-stranded attP site.

Recombineering oligos were designed to insert a viral att site at the desired location in the genome. Oligos included the final 70 bases of the gene of interest (before the stop codon), the att site (sequence: GGTTTGTCTGGTCAACCACCGCGGTCTCAGTGGTGTACGGTACAAACC), and the 70 bases following, including the stop codon. Directionality of the oligo was chosen based on the location of the gene of interest in either the right or left replicore, so that the oligo could be treated as an Okazaki fragment and incorporated into the genome on the lagging strand.

Checking PCR

Verification of transformations was done via PCR. PCR cycling protocol was as below. Annealing temperature was calculated via the NEB Tm calculator.

95° C	30 s
95° C	30 s
[annealing temp]	30 s
72° C	[1 minute for each kb]
68° C	2-5 min
10° C	hold

Curing to Remove Recombineering Plasmid

In order to minimize unintended effects of the recombineering plasmid, isolates that were verified to have been successfully tagged were grown up in 5 ml cultures and plated on 7H10 plates with 15% sucrose. Colonies that grew were then re-streaked on a kanamycin and hygromycin plate. Those that grew on hygromycin but not kanamycin were determined to be lacking the recombineering plasmid, and were grown in culture and stored as glycerol stock for future use.

***M. smegmatis* Growth in Hypoxia**

Growth in hypoxia was conducted based on the Wayne model (Wayne & Hayes, 1996). 10 ml liquid cultures were grown under standard conditions to an OD indicating exponential phase, and then diluted to an OD of 0.01. 36.5 ml of diluted culture was added to a bottle and sealed with a rubber cap, metal cover, and a clamp. The bottles were then secured and incubated at 37° C, shaking at 125 rpm, for 42 hours.

Total Protein Extraction

Each 36.5 ml culture was grown under desired conditions (standard, hypoxia, etc.) to the desired OD. Cultures were pelleted in 50 ml conical tubes using the Eppendorf Centrifuge 5810R at 4° C for 5 minutes at 3900 rpm. After removal of supernatant, pellets were washed 2x with 7H9 with Tween 80 and glycerol, but no ADC components. The final pellets were then suspended in 1 ml PBS + 2% SDS with Amresco protease inhibitor cocktail added to 1x immediately before use. Resuspended pellets were transferred to a lysing matrix B tube and placed into an MP FastPrep-24™ 5G and bead-beated 4x at 6.5 m/sec, 30 seconds per cycle, with 1-minute incubations on ice in between cycles. Tubes were then spun at 13,000 rpm in an Eppendorf™ 5424 Microcentrifuge. Supernatant was removed, placed in a new screw cap tube, and stored at -20° C.

Western Blotting

Western blotting was conducted using Mini-PROTEAN TGX Precast Gels with 50 µl wells. Lysates were heated at 95°C for 10 minutes again upon removal from storage. Gels were run according to manufacturer instructions, loading 10 µl of Bio-Rad Precision Plus Dual Color Ladder and 20-35 µl of lysate combined with 4x sample buffer (20 mM Tris-Cl at pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol). Gels were run at 140 V using the Bio-Rad Mini-PROTEAN Tetra System. Blotting was conducted using PVDF membrane, and the machine was run at 100 V for one hour. Blots were then stained using Ponceau S before blocking and imaged on a Bio-Rad Gel Doc XR. They were then blocked using TBS + 3% nonfat milk for

30-60 minutes, then incubated with primary antibody (Monoclonal ANTI-FLAG M2 antibody, Sigma, F1804-200UG, 1 mg/ml stock, 15 μl in 15 ml blocking solution) overnight at 4°C. They were then incubated with secondary antibody (Sigma anti-mouse whole molecule IgG produced in rabbit, 1 μl in 30 ml blocking solution) for 30 minutes at room temperature, washed in TBS + Tween 20, and then imaged on a Bio-Rad ChemiDoc XRS using the Pierce ECL Plus Western Blotting Substrate kit.

Table 2: Oligos

Oligo name	**Function**	**Sequence**
SSS585	Forward primer for checking junction between genome and rraA gene (used as a control to verify amplification of genomic DNA)	CAGCCAGAATGGCAGAGCAG
SSS586	Reverse primer for checking junction between genome and rraA gene	AAGACCATCCGTGCCGGCAA
SSS1061	Forward primer to verify presence of 1.5kb segment #1 of pKM444 or pKM461	CATCCTGACGGATGGCCTTT
SSS1036	Reverse primer to verify presence of 1.5kb segment #2 of pKM444 or pKM461	CGGATGACTACCAGGGCT
SSS1037	Forward primer to verify presence of 1.5kb segment #2 of pKM444 or pKM461	CTTCGAGATGAGAGCCCTG
SSS1038	Reverse primer to verify presence of 1.5kb segment #2 of pKM444 or pKM461	CGCTAGTTAACCTACGACATC
SSS980	Oligo to incorporate attP into rne (msmeg_4626).	GTGACCAGCGGGTAGAGGGTCAAACTGGCC CCCCCCTGGATCTCGCCTGAGACACCGTGAT GCTCGTCTAGGTTTGTACCGTACACCACTGA GACCGCGGTGGTTGACCAGACAAACCGTCGT GGCTGGGCGGCCCCGCCGGACGTGCCGCGG CCCGGCGGCGCACCCGTGCGCGCGGCGCCG TCACC

SSS981	Oligo to incorporate attP into a putative RNA helicase gene (msmeg_1540).	CGCACGTGGTGGTGCGCACCGGGGTGGACG CGGCCGCAGGCCGCGCCGCTCCAACGAGTC GTCCGCGCGCGGTTTGTCTGGTCAACCACCG CGGTCTCAGTGGTGTACGGTACAAACCTAGA GACGCGCGAAATGTGCGGTCCCACCCGACCT CTCCATACCGGGTGGGACCGCACAGGCATTT GTGG
SSS982	Oligo to incorporate attP into the RNase J gene (msmeg_2685).	AGGTGCTCAAGCTCGCGGGCCTGTTCACCGG CGGGATCCTGCTGCTGGCGGGTTGAGCTCAG ATCGCTCAGGTTTGTACCGTACACCACTGAGA CCGCGGTGGTTGACCAGACAAACCGATCTCTA TGACGGTCGGGACGATCATCGGCTGCCTGCG GTAGGTCTCGCCCACCCACTTGCCGACCGTG
SSS1008	Forward primer for L junction to check incorporation of Orbit plasmid following msmeg_2684. To be used with SSS1009.	CAGAAGGTTGAGCGCGAA
SSS1009	Reverse primer for L junction to check incorporation of Orbit plasmid.	CCTGGTATCTTTATAGTCCTGTCG
SSS1010	Forward primer for R junction to check incorporation of Orbit plasmid.	TGCACGGGACCAACATCTTCGTGG
SSS1011	Reverse primer for R junction to check incorporation of Orbit plasmid following msmeg_2684. To be used with SSS1010.	TGTCGCGTTGGAGGTGCTCAA
SSS623	Forward primer near end of msmeg_4626 to check incorporation of Orbit plasmid. To be used with SSS1009.	ATCGACGAGCAGCTCGCGAA

SSS1090	Reverse primer for R junction to check incorporation of Orbit plasmid following msmeg_4626. To be used with SSS1010.	CTTGCCGCCGGTCTTGACGA
SSS1097	Forward primer for L junction to check incorporation of Orbit plasmid following msmeg_1540. To be used with SSS1009.	TTCGGTGAGCCCATTCGC
SSS1098	Reverse primer for R junction to check incorporation of Orbit plasmid following msmeg_1540. To be used with SSS1010.	AACGCGGTGCGTGACGGGT
SSS1099	Reverse primer to pair with SSS1097: post-msmeg_1540 genomic DNA. To provide positive confirmation of a missing insert if necessary.	TGTTCAGCCTCGGCAACG

Results

Strain construction

Three strains of *M. smegmatis* were constructed via a novel recombineering-based approach developed by Kenan Murphy (UMass Medical School) and diagramed in Figures 1 and 2. Proteins were tagged at the C-terminal end with FLAG and DAS+4 (McGinness et al., 2006). The proteins tagged were RNase J (msmeg_2685), RNase E (msmeg_4626), and a putative helicase (msmeg_1540); these proteins were chosen as candidates because they are known or suspected to be involved in RNA degradation, and none of them appears to be part of an operon. The absence of an operon is significant because an entire plasmid is inserted into the genome following the gene, with potential to disrupt downstream genes if they are co-transcribed with genes upstream of the insertion or regulated by elements upstream of the insertion.

Each strain was evaluated for successful incorporation of the plasmid by PCR. The primers constructed for PCR were designed to amplify the portion of DNA surrounding the junctions both upstream and downstream of the site of recombination. PCR products were then purified and sequenced. For the RNase J strain, both junctions were confirmed, while for the RNase E and helicase strains, only the downstream junction was confirmed. PCR was attempted for the upstream junctions for these strains several times, using several sets of primers annealing to different positions, but none of these yielded bands.

Though the presence of the tag was verified, the RNase J strain did not yield a specific band on a western blot, under any conditions. The helicase strain produced a faint specific band (Figure 4). The RNase E strain showed a strong specific band (Figure 3), suggesting that this strain could be used to assess condition-dependent changes in RNase E protein levels.

RNase E protein abundance appears to be decreased in hypoxic cultures

To determine if RNA degradation enzymes are downregulated in quiescent *M. smegmatis*, we compared RNase E abundance in log phase and hypoxic cultures. Two isolates of the RNase E strain were tested, with two biological replicates for each isolate in each condition. Each isolate originated from a distinct colony on a transformation plate, and both had been "cured" by counterselection on sucrose to ensure absence of the recombineering plasmid. Cultures were grown under aerobic and hypoxic conditions, and the cells were lysed during mid-log phase for the aerobic cultures, and 42 hours after sealing the bottles for the hypoxic cultures. Unpublished data from experiments performed by other lab members indicate that growth ceases approximately 24 hours after sealing the bottles. Lysate was used for total protein quantification and western blotting. When loading the gel for blotting, the bacterial lysates were normalized based on protein concentration, in order to ensure that the same quantity of total protein was being loaded in each well.

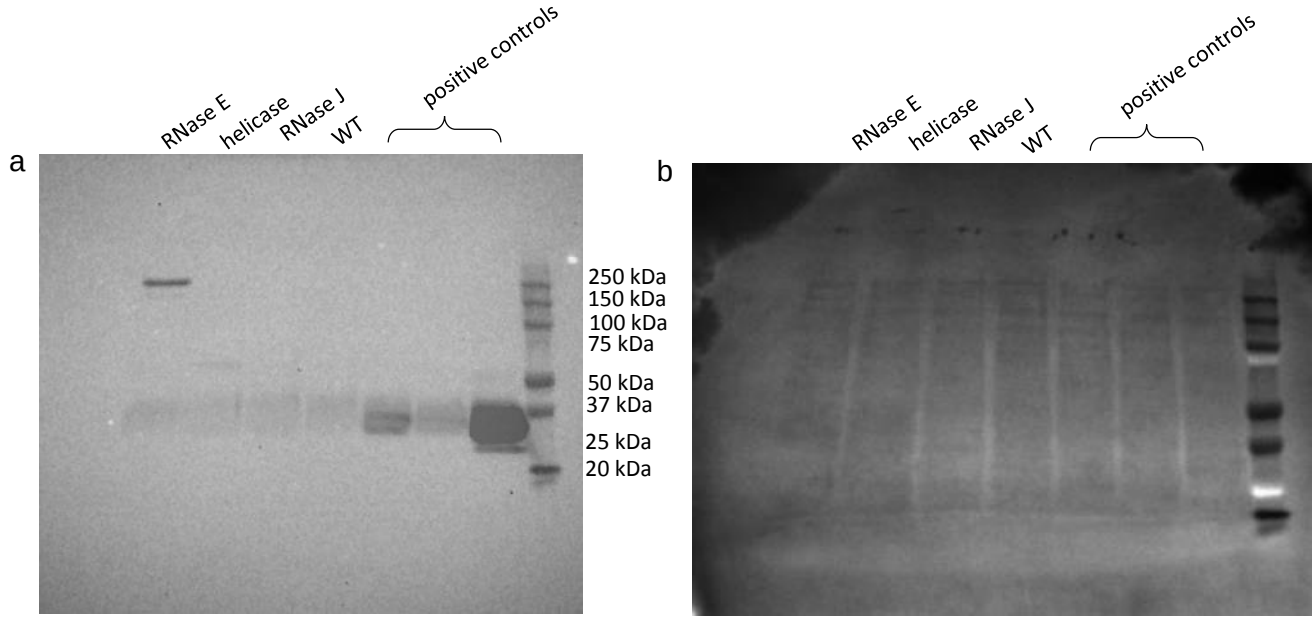

Figure 3: Western blotting reveals a specific band for the RNase E-FLAG strain. All three tagged strains were grown in aerobic conditions. (A) Anti-FLAG western blot. Image including ladder has been overlaid. Positive controls were acquired from Christina Baer at UMass Medical School. (B) Ponceau S staining to verify equal total protein loading. Expected size for positive controls (Venus fluorescent protein, three different expression levels) was 27 kDa. Expected sizes for experimental proteins were 60 kDa for RNase J, 112 kDa for RNase E, and 49 kDa for helicase.

When normalized to the optical densities of the cultures from which they were derived, the total protein concentrations were modestly decreased in hypoxia compared to log phase (Figure 4). This suggests that protein content per cell may be slightly lower in hypoxia. However, the decrease only reached statistical significance for one of the three strains.

On the western blot (Figure 5), the bands from the hypoxic cultures were substantially lighter than those from the log phase cultures. This reduction of band intensity in hypoxia persists regardless of discrepancies in apparent protein load (Figure 5B). As the blot was normalized to total protein level and hypoxic cultures were found to have decreased protein concentrations, the tagged protein appears to comprise a decreased proportion of the total protein present in the cultures, and fewer copies of the protein per cell are present.

A second band also appeared below the first, with a faint presence in the lysates derived from log phase cultures and a much stronger presence in those from hypoxic cultures.

A similar experiment was done with the helicase-tagged strains, but the experimental blot comparing hypoxic and aerobic conditions was inconclusive, with only a faint band appearing for the aerobically grown strain in the image resulting from an hour-long exposure (data not shown). Because the signal from the aerobic condition was so weak, we could not conclude with confidence that the signal from the hypoxic samples was in fact decreased.

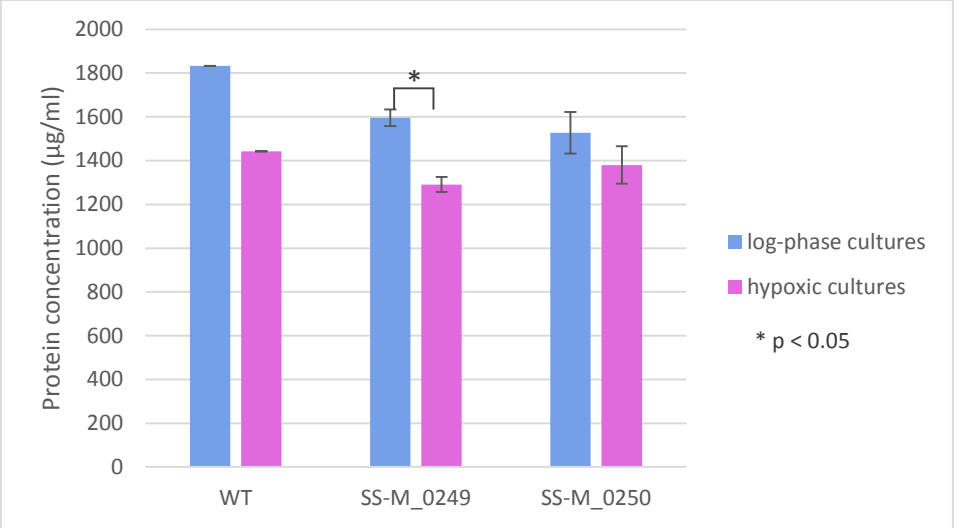

Figure 4: Comparison between normalized protein concentrations in log phase and hypoxic cultures, including wild type strains and the two RNase E tagged isolates, SS-M_0249 and SS-M_0250. Two-sample T test assuming equal variance. Error bars represent standard deviation.

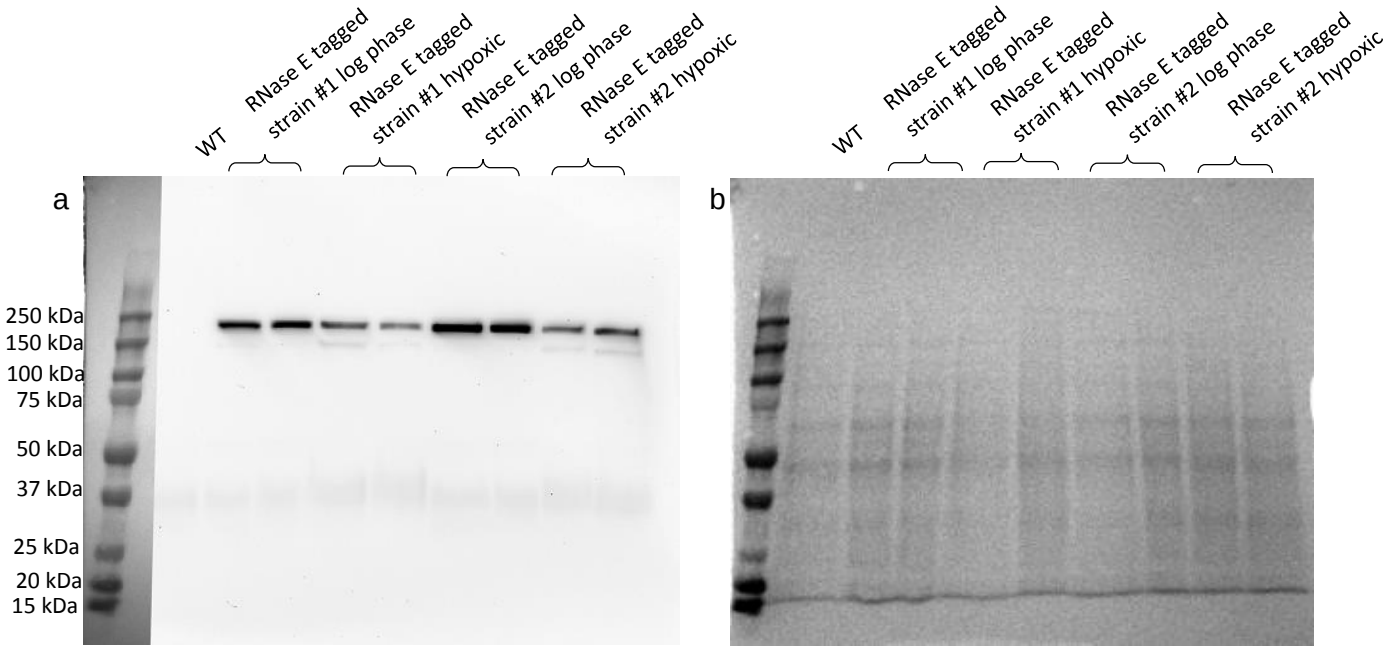

Figure 5: RNase E protein abundance appears to be reduced in hypoxia. (A) Western blot with RNase E tagged strains in hypoxia. 8-minute exposure is shown. A negative control (unmodified mc²155) and two isolates of each strain were analyzed, with two replicates of each strain in each experimental condition. (B) Ponceau S staining confirms that the differences in panel A cannot be explained by unequal protein loading. Expected size for RNase E is 112 kDa.

Discussion

The RNase E tagged strain appears to show some downregulation of RNase E protein under hypoxic conditions, which would provide evidence supporting the hypothesis that regulation of RNA degradation proteins affects mRNA half-lives in quiescent mycobacteria. The appearance of a second, smaller band that is stronger in hypoxia even suggests a possible mechanism of protein regulation: cleavage of proteins could result in a smaller protein that has altered properties but still shows up on a western blot.

Though the potential implications of the results are noteworthy, the failure to confirm the upstream junction of the tag is a serious concern. Since the adjustment of primers and PCR conditions yielded no results, poor PCR quality is unlikely. The size of the protein that appears on the western blot is also a concern, though aberrant migration of RNase E (specifically, running as a larger protein) has been previously reported in *M. tuberculosis* (Zeller et al., 2007) and *E. coli* (Casaregola et al., 1994). According to Caseregola et al., the discrepancy in the *E. coli* protein may be due to the high quantity of highly charged residues in the C-terminal region of the protein. The *M. smegmatis* RNase E appears to have this same pattern of many highly charged residues in this area. However, some follow-up work in process (currently being conducted by other members of the Shell lab) suggests that attempts to incorporate a plasmid following the RNase E-encoding gene results in a large (3kb or greater) duplication of DNA between the gene of interest and the incorporated plasmid. Based on this information, it is currently impossible to come to a conclusion regarding what is being visualized on the gel.

The intent of the Shell lab is to continue moving forward with this research. Based on the assumption that tagging RNase E at the C terminal end (the present technique) yields problematic results, further research may begin with tagging at the N terminal end.

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