Exploring a Transcriptional Regulatory Region in Mycobacteriophage JacoRen57

Ali Almail, Elizabeth Heeg , Byron Noordewier, Sara S. Tolsma
Northwestern College, Department of Biology, Orange City, IA 51041

SEA PHAGES
hhmi Howard Hughes Medical Institute

Abstract

Mycobacteriophages are infectious particles that infect mycobacteria, and little is known about cis-regulatory elements that control their gene expression. In phage genomes, cis-regulatory elements commonly precede a series of genes that are expressed as an operon. JacoRen57 is a cluster AB mycobacteriophage that possesses forward and reverse genes with non-coding gaps interspersed throughout its genome. We assayed one of the gap regions of JacoRen57 (40644-40974 bps) for regulatory activity in the downstream direction when present in its host, *Mycobacterium smegmatis*, by cloning the region into pLO86 – a vector containing the mCherry reporter gene. The putative regulatory region induced the expression of mCherry *in vivo*, indicating the presence of a promoter in this region of the JacoRen57 genome. Utilizing 5' deletion analysis, we identified transcriptional repressor and activator elements within this regulatory region. We are conducting further experiments to understand the characteristics of the transcriptional repressor region and which sigma factor(s) bind(s) to this regulatory region.

Introduction

Mycobacteriophages are viruses that infect bacteria from the genus *Mycobacterium* through the lytic and lysogenic cycles (Clokie et al., 2011). JacoRen57 is a *Siphoviridae* morphotype, lytic, and cluster AB mycobacteriophage that infects *Mycobacterium smegmatis* mc²155 (Phagesdb, 2021). Its double stranded DNA genome is 70,300 bps, consisting of 73 genes, 33 of which are forward genes followed by 16 reverse genes and 24 forward genes from the right to left arm of its genome (NCBI:MK279840.1, 2019).

The second transition from reverse to forward genes (40644-40974 bp) possesses a 331 bp region with minimal coding potential. The gene directly downstream of this gap is a RecA-like DNA recombinase, which functions in phage DNA replication, genome packaging, generation of genomic diversity, and dsDNA repair (NCBI:MK279840.1, 2019; Murphy, 2012). There are two genes proceeding the RecA-like DNA recombinase that overlap with each other, suggesting the expression of the three genes in the same operon (NCBI:MK279840.1, 2019). Utilizing online promoter predictor softwires (PePPER, BPROM, Neural Network Promoter Predictor), we found matches putative matches to promoter sequences.

Regulatory regions in prokaryotes and phages follow an operon model, where a regulatory region precedes a series of genes. A regulatory region typically consists of a promoter and a repressor/operator region Additionally, various sigma factor consensus sequences have been characterized in *Mycobacterium tuberculosis*, which is in the same genus as *M. smegmatis*. Many of the characterized sigma factor sites are responsive to cell stress signals (Manganelli et al., 2004).

To assess regulatory activity of a DNA sequence, fluorescent reporter genes constructs, pLO86 and pLO87, were used to assess promoter activity of the JacoRen57 putative regulatory region which we refer to as 51. pLo86 lacks a promoter region, but pLo87 possesses an hsp60 promoter (Oldfield et al., 2014). These plasmids encode the mCherry gene, a reporter gene that codes for a pink fluorescent protein which is observable under visible light conditions. If the cloned sequence functions as a promoter *in vivo*, then the mCherry gene will be transcribed and translated to produce mCherry. However, if the cloned sequence does not function as a promoter *in vivo*, then mCherry will not be expressed.

In this study we utilized reporter gene assays with pLO86 and pLO87 to assess the activity of the putative regulatory region (40644-40974 bp) in JacoRen57. We demonstrate that the putative regulatory region consists of three elements, a repressor region and two promoter elements that function in the downstream direction. We are in the process of conducting more experiments to elucidate the trans-acting elements of this regulatory sequence.

References

Almail,A., Dorhout,K.E., Johnson,J., Jorgensen,H.J., Tolsma,S., Garlena,R.A., Russell,D.A., Pope,W.H., Jacobs-Sera,D. and, & Hatfull,G.F. (2019). *Mycobacterium phage JacoRen57, complete genome* (1547005285). NCBI Nucleotide Database. http://www.ncbi.nlm.nih.gov/nuccore/MK279840.1
Clokie, M. R., Millard, A. D., Letarov, A. V., & Heaphy, S. (2011). Phages in nature. *Bacteriophage*, *1*(1), 31–45. https://doi.org/10.4161/bact.1.1.14942
Manganelli, R., Dubnau, E., Tyagi, S., Kramer, F. R., & Smith, I. (1999). Differential expression of 10 sigma factor genes in Mycobacterium tuberculosis. *Molecular Microbiology*, *31*(2), 715–724. https://doi.org/10.1046/j.1365-2958.1999.01212.x
Manganelli, Riccardo, Proveddi, R., Rodrigue, S., Beaucher, J., Gaudreau, L., & Smith, I. (2004). σ Factors and Global Gene Regulation in Mycobacterium tuberculosis. *Journal of Bacteriology*, *186*(4), 895–902. https://doi.org/10.1128/JB.186.4.895-902.2004
Murphy, K. C. (2012). Phage recombinases and their applications. *Advances in Virus Research*, *83*, 367–414. https://doi.org/10.1016/B978-0-12-394438-2.00008-6
Oldfield, L. M., & Hatfull, G. F. (2014). Mutational analysis of the mycobacteriophage BPs promoter PR reveals context-dependent sequences for mycobacterial gene expression. *Journal of Bacteriology*, *196*(20), 3589–3597. https://doi.org/10.1128/JB.01801-14
Phagesdb. (2021). *The Actinobacteriophage Database | Phage JacoRen57*. https://phagesdb.org/phages/JacoRen57/
Valero-Rello, A., López-Sanz, M., Quevedo-Olmos, A., Sorokin, A., & Ayora, S. (2017). Molecular Mechanisms That Contribute to Horizontal Transfer of Plasmids by the Bacteriophage SPP1. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.01816

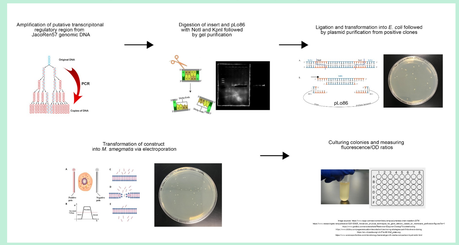
Acknowledgements

I would like to thank Dr. Tolsma, Dr. Heeg, and Dr. Noordewier for your wisdom and supervision. I would like to thank the Hatfull Lab at the University of Pittsburgh for the provision of the pLO86 and pLO87. Support for this project was provided by Northwestern College and Howard Hughes Medical Institute . I would also like to thank the directors of the NWC Honors Program Dr. Feenstra and Dr. Vonder Bruegge for the research opportunity.

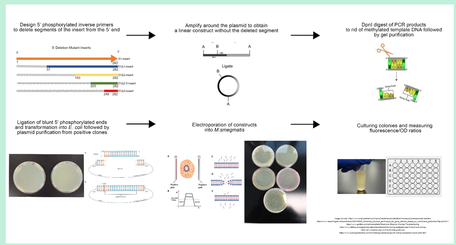
QR Code to Access Detailed Materials and Methods

Materials and Methods

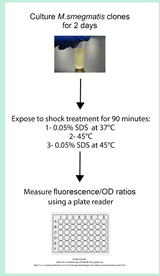
Cloning the Transcriptional Regulatory Region into pLo86



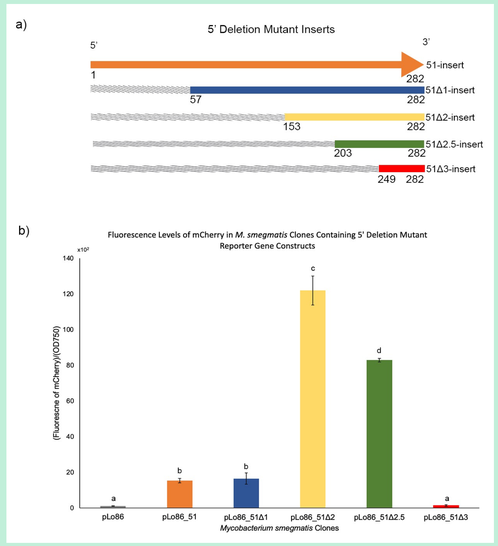
Construction of 5' Deletion Mutants



Shock Assays



Results



5' Deletion Assays:

We constructed 5' deletion mutants to identify elements of the regulatory region that modulate gene expression. We found that the deletion of bases 57-152 resulted in a significant increase of the fluorescence ratio of mCherry compared to pLo86_51 and pLo86_51 Δ1, indicating increased mCherry expression (Figure 1). We also found that the the deletion of bases 153-202 resulted in a significant decrease in the fluorescence of mCherry compared to pLo86_51 Δ2, which indicates a decrease in mCherry expression (Figure 1).

Figure 1 (a): Diagram of the 5' deletion mutants of the transcriptional regulatory region of JacoRen57. The dashed regions represent deleted segments. (b): mCherry fluorescence ratios of M. *smegmatis* containing pLo86 constructs with the 5' deletion mutant inserts. We had biological triplicates for each clone. Different letters indicate significant difference between clones ($p<0.05$). Error bars represent standard deviation.

PCR to Confirm 5' Deletion Mutants:

We designed primers that annealed to plasmid sequences outside the transcriptional regulatory region to confirm the 5' deletion mutations. If the 5' deletion mutants were successful, we expected the following band sizes: pLo86= 234 bp, pLo86_51= 516 bp, pLo86_51Δ1= 460 bp, pLo86_51Δ2= 361 bp, pLo86_51Δ2.5= 314 bp, pLo86_51Δ3= 268 bp. We obtained the expected band sizes for each respective deletion (Figure 2).

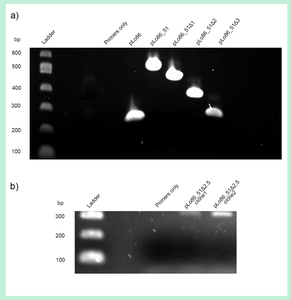


Figure 2: (a) 1% agarose gel of PCR products to confirm the 5' deletion mutations of pLo86_51, pLo86_51Δ1, pLo86_51Δ2, and pLo86_51Δ3. (b) 1% agarose gel of PCR products from colony verification to confirm the 5' deletion mutation of pLo86_51Δ2.5. In both (a) and (b), we used the NEB 1kB plus ladder.

Shock Assays:

Given what we know about sigma factor expression in *M. tuberculosis* under different conditions (Manganelli et al., 1999), we attempted to expose *M. smegmatis* with pLo86_51 and pLo86_51 Δ2 to shock treatments to narrow down which sigma factors bind to the transcriptional regulatory region. *M. smegmatis* containing pLo87 served as an experimental control to confirm if the observed mCherry fluorescence ratio changes were unique to our transcriptional regulatory region. This experiment assumes that increased sigma factor expression can lead to increased reporter gene expression. We found that heat shock at 45°C significantly decreased the fluorescence ratio of mCherry compared to a non-treatment control in both pLo86_51 and pLo86_51 Δ2, indicating a decrease in reporter gene expression (Figure 3). In both these clones, we found that SDS treatment at 45°C significantly increased the fluorescence ratio of mCherry significantly, indicating an increase in mCherry gene expression (Figure 3). None of the treatments resulted in significant differences between the mCherry fluorescence ratios in *M. smegmatis* transformed with pLo87 (Figure 3).

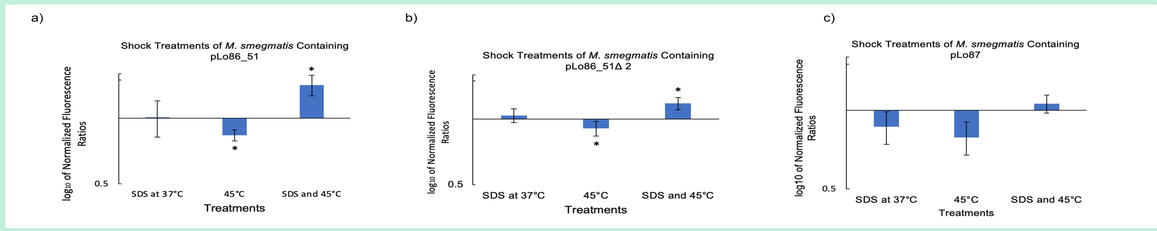


Figure 3 : We exposed *M. smegmatis* to shock treatments to narrow down the possible interacting sigma factors. Each of the clones in (a), (b), and (c) were treated with 0.05% SDS at 37°C, 45°C, and 0.05% SDS at 45°C. The experiment was replicated sixfold for each clone. Results are graphed on a log scale of a ratio of the fluorescence levels of mCherry at the treatment condition over the control. Values above 1 indicate an increase in fluorescence ratios, while values below 1 indicate a decrease in fluorescence ratios. * indicates a significant difference from the no treatment control at 37°C ($p<0.05$). The error bars represent percent deviation from the sample's mean ratio.

Discussion

To identify the transcriptional regulatory elements within this region, we constructed 5' deletion mutants . We successfully constructed four deletion mutants, as we obtained the expected band sizes from PCR confirmation (Figure 2). We used the constructs to conduct reporter gene assays to identify which segments of the transcriptional regulatory region modulated reporter gene expression. We found that:

1. The deletion of bases 57-152 resulted in a significant increase in the fluorescence ratio of mCherry compared to all other clones, indicating the increase in mCherry gene expression. This supports the presence of a transcriptional repressor element (Figure 4).
2. The deletion of bases 153-202 in pLo86_51Δ2.5 resulted in a significant decrease in fluorescence ratio of mCherry compared to pLo86_51Δ2, indicating a decrease in mCherry expression. This supports the model that there are promoter elements within 153-202 bp and within 203-249 bp, as 249-282 bp did not modulate mCherry gene expression (Figure 1 and 4).

To narrow down which sigma factors interact with the transcriptional regulatory region, we conducted the heat shock assays. We found that 45°C treatment resulted in a significant decrease in the fluorescence ratio of mCherry in pLo86_51 and pLo86_51 Δ2 (Figure 3). This led us to hypothesize which sigma factors may be interacting with our regulatory region based on studies by Manganelli et al. (1999) that measured which sigma factors were downregulated in *M. tuberculosis* due to heat shock. The closest consensus binding sequence present in our regulatory region is that of sigG (Figure 4). Interestingly, SDS treatment at 45°C resulted in an increased ratio of mCherry fluorescence (Figure 3). This may support some synergistic interactions between two sigma factors that modulate gene expression independently. We had previously found a sigE consensus binding site in the transcriptional region that had a larger spacing between the -35 and -10 elements than the published consensus in *M. tuberculosis* (Figure 4), and we hypothesize that this could be the synergistic sigma factor. We do not suspect that the transcriptional repressor region is necessary for this synergistic activity, as the absence of the repressor element resulted in a significant increase with combined SDS and heat shock treatments. We plan on conducting this assay with the recently constructed pLo86_51Δ2.5.

The 5' deletion assay data supports the model that there is a transcriptional repressor region and two promoter elements. Furthermore, sequence comparison to published sigma factor binding sites in *M. tuberculosis* support the presence of two promoter elements that bind different sigma factors (Figure 4). We hypothesize that promoter element 1 binds sigE and promoter element 2 binds sigG (Figure 4). We are in the process of conducting site-directed mutagenesis to confirm if the hypothesized cis-acting elements of sigE and sigG modulate gene expression. We also plan to send the remaining constructs for sequencing to confirm the absence of mutations (note: we used a high-fidelity DNA polymerase to construct all expression constructs).

These results present interesting implications for phage gene expression regulation. This transcriptional region precedes an operon of three overlapping genes, one of which is a RecA -like DNA recombinase (NCBI:MK279840.1, 2019). Given that horizontal gene transfer is common between the host and phage genome, it is possible that this regulatory region resulted from multiple horizontal gene transfer events that later accumulated mutations (Valero-Rello, 2017). These regulatory regions were possibly maintained since neither of these consensus sequences may represent ideal binding sites for sigma factors, so maintaining both sigma factor binding sites resulted in higher levels of gene expression than is possible with only one promoter element, which is supported by our data and also provides alternative modes of gene expression regulation that are context dependent (Figure 1). Further research is needed to understand how this fits into models of phage evolution, to explore the binding site and transcpiriotnal repressor protein that binds to the transcriptional repressor element, and why RecA–like DNA recombinase expression would need to be regulated by such a regulatory region.

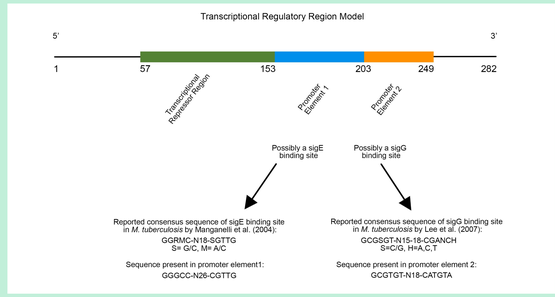


Figure 4: Proposed model of the transcriptional regulatory region. This model contains a repressor region (57-153 bp), a promoter element (153-203 bp), and another promoter element (203-249). The possible hypothetical binding site of sigE in promoter element 1 and sigG in promoter element 2 are listed.