Analysis of a Putative Promoter in Mycobacteriophage JacoRen57

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Abstract

JacoRen57 is a cluster AB mycobacteriophage that infects *Mycobacterium smegmatis* mc²155. We recently reported on the characterization of a putative promoter in JacoRen57 using an mCherry reporter construct. This promoter is present in a gap upstream of a gene that is present in all AB phages. In all cases, these are forward genes immediately following a long series of reverse genes. The genes are most frequently identified as a RecA-like DNA recombinases but also as RepA by bioinformatics. To further analyze this putative promoter and gene product, NWC Molecular Genetics students cloned the RecA-like DNA recombinase into an *E. coli* expression vector with a TVMV removable N-terminal His-tag. They expressed and we purified the tagged protein and are using it to immunize Balb/c mice. We plan to use the antiserum to confirm RecA-like DNA recombinase expression patterns when JacoRen57 infects *M. smegmatis*.

Introduction

NWC alum, Ali Almail, characterized a putative promoter in the novel bacteriophage, JacoRen57 (manuscript in preparation). This previous work indicates the presence of a promoter between genes 49 and 50. Based upon bioinformatics, gene 50 was identified as a RecA-like DNA recombinase. RecA is a protein common to bacterial genomes. RecA functions in homologous recombination and aids in DNA repair (Del Val, et al., 2019). We are collaborating with Dr. Heeg's Molecular Genetics students to understand this promoter and the gene it regulates better. Promoters in bacteriophages are not well studied. Our experiments will contribute to the evidence that a promoter has indeed been identified, and will go on to investigate how that promoter functions within the genome of JacoRen57. We intend to generate antibodies against the protein encoded by this gene using BALB/c mice. Anti-RecA-like recombinase antibodies will be a tool to identify the protein product of gene 50, confirming our bioinformatics analysis. Additionally, our work will be the first to demonstrate transcription and translation of gene 50 in the context of a JacoRen57 of *Mycobacterium smegmatis*. We hope to confirm the expression patterns observed when this promoter controlled the expression of the mCherry reporter gene.

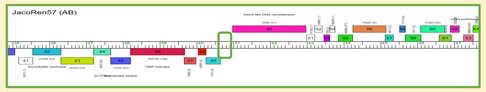

Figure 1. Phamerator map of the portion of the JacoRen57 genome that includes the putative promoter we are analyzing. The putative promoter is boxed in green and we believe it regulates the expression of Gene 50 (pink) and possibly additional downstream genes in an operon. Gene 50 was identified as a RecA-like DNA Recombinase by bioinformatics.

Materials and Methods

SDS-PAGE protocol: We ran phage high titer lysates (HTL) on an SDS-PAGE gel to isolate proteins from the phages. To prepare samples, we boiled high titer lysates with equal parts Laemmli buffer for 10 minutes. Samples were loaded onto the gel and then individual bands were cut out, eluted overnight, and concentrated and purified using a Pierce Concentrator (Thermo Scientific). BCA protein assay kit was used to determine the concentration of the proteins from the gel.

Injection protocol: Each mouse (BALB/c) was injected with roughly 50-100μl of the following immunization: 50μl of HTL or phage protein + 150μl PBS + 200μl incomplete Freund's adjuvant for the primary immunization. Complete adjuvant was used for boosters. Two mice received immunizations with whole JacoRen57 HTL and two mice were injected with one band from RedRaider77.

Collecting serum protocol: Serum was collected by tail bleeding the mice. We hole punched the ear of one mouse from each cage to differentiate the samples we collected. The serum was incubated for 1 hour at 37 degrees Celsius and then stored in the fridge overnight. Next, the serum was spun, and the supernatant containing the antibodies was stored at 4 degrees Celsius.

ELISA: Two ELISAs were performed. The first used JacoRen57 HTL as the antigen, and the primary antibody was anti-JacoRen antiserum. The second tested for cross reactivity between phages, and we used Roots, Wrigley, KEBS, Stormbreaker8, and Deadpool HTLs as the antigens. The primary antibody was anti-Roots. Secondary antibody in both cases was rabbit-anti-mouse.

Western blot: We ran 5 western blots with the following primary antibodies: anti-CBorch11 polyclonal, anti-Roots band 1, anti Sibs6, anti-JacoRen57 polyclonal, and a negative control. Secondary antibodies were rabbit-anti-mouse IgG (Invitrogen). The gels from which we ran the western blots contained 15 phages, 10 were Smeg phages, 2 were Terrae phages, and 2 were Folio phages.

Figure 2. Research workflow. This is a collaborative research project. Cloning is being done by the NWC Molecular Genetics students. Immunization is being done by the NWC Immunology students.

ELISA–Enzyme-linked ImmunoAssay Analysis

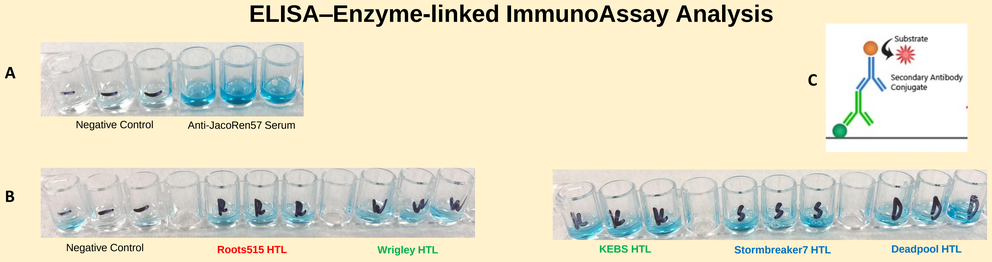

Figure 3. ELISA Assays. A: Antigen is JacoRen57 high titer lysate. Primary antibodies are indicated. Secondary antibody is HRP-conjugated rabbit anti-mouse antibodies. A positive ELISA Assay indicates that our JacoRen57 HTL-immunized mice are making anti-JacoRen57 antibodies. B: Antigen is Roots515 high titer lysate Primary antibodies are indicated. Secondary antibody is HRP-conjugated rabbit anti-mouse antibodies. Polyclonal antibodies against a single protein isolated from Mycobacteriophage Roots515 recognize antigens from Mycobacteriophages, Gordonia phages, and Microbacterium phages indicating biochemical similarity between phages that infect different hosts. C. Schematic representation of our ELISA Assay.

Western Blot Analysis

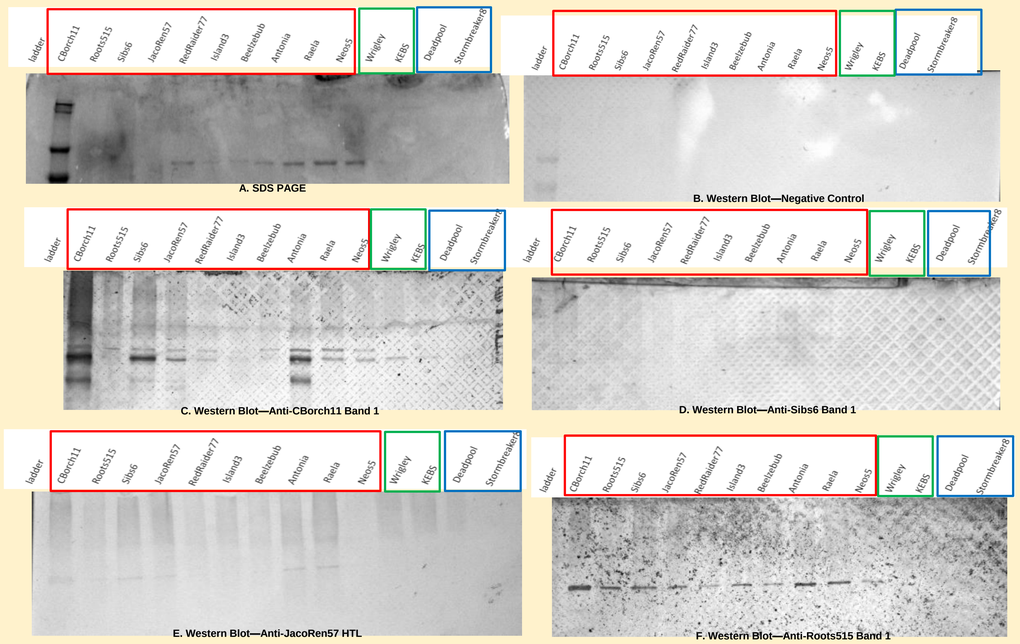

Figure 4. Western blot analysis of 14 different Actinobacteriophages probed with serum from mice immunized with various antigens indicate that even though the phages infect different hosts they are biochemically similar enough to be recognized by antibodies raised against proteins from Mycobacteriophage. The red boxes indicates *M. smegmatis* phages. The green boxes correspond to *G. terrae* phages, and the blue boxes corresponds to *M. foliorum* phages. A. SDS-PAGE for loading comparisons. B. Negative control. C. Western blot probed with anti-CBorch11 Band 1 antiserum. D. Western blot probed with anti-Sibs6 Band 1 antiserum. E. Western blot probed with anti-JacoRen57 high titer lysate antiserum. F. Western blot probed with anti-Roots515 Band 1 antiserum.

Citations
Del Val E, Nasser W, Abaibou H, Reverchon S. RecA and DNA recombination: a review of molecular mechanisms. Biochem Soc Trans. 2019 Oct 31;47(5):1511-1531. doi: 10.1042/BST20190558. PMID: 31654073.
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. Accession number MK279840.1. https://www.ncbi.nlm.nih.gov/nuccore/MK279840.

Discussion

We have been working with the NWC Molecular Genetics students in a collaborative research project. We are working to further analyze a putative promoter identified in the mycobacteriophage JacoRen57. Our work is important to confirm the work previously done in which the putative promoter was analyzed using a reporter gene. We hope our work will demonstrate that the promoter functions in its natural context—the phage JacoRen57 and regulating the production of RecA-like DNA recombinase—in the same way it functioned when it regulated the mCherry reporter gene. While the Molecular Genetics students work on cloning the RecA-like DNA recombinase, we have been learning to immunize mice, collect blood for serum by tail-bleeding, analyzing serum for the presence of specific antibodies by ELISA and Western Blot analysis. We have also been working to isolate specific proteins from bacteriophages to use as immunogens. We will be presenting our work at the national SEA-PHAGES Symposium and the Iowa Academy of Science annual meeting at the University of Northern Iowa.

We have successfully:

- Immunized mice
- Collected serum by tail bleeding
- Performed Western blots, probing them with serum from our immunized mice
- Performed ELISA assays to demonstrate that our immunized mice have responded to our immunogens

Conclusions:

- Antibodies raised against mycobacteriophages also recognize Microbacterium phages and Gordonia phages
- The mice we immunized with JacoRen57 HTL are making anti-JacoRen57 antibodies

Future Directions

In our future work, we will follow our current protocols of immunization and sera collection. We will immunize our mice using the JacoRen57 protein cloned by the Molecular Genetics students and collect the antiserum they generate against the protein .These antibodies will allow us to confirm that RecA is expressed following the same pattern observed by Almail in his previous research.

The antiserum generated by our mice is polyclonal in nature, meaning that it contains a variety of antibodies that are specific for different epitopes of the immunizing antigen. It is possible to produce monoclonal antibodies by fusing and cloning B cells with myeloma cells. We plan to attempt to generate monoclonal antibodies using the spleen cells of our mice once we have collected enough polyclonal sera for the analyses we have planned.

The antibodies generated in our experimentation can be used in the future to answer questions regarding phage biology and expand understanding of bacteriophages in general.

Acknowledgements

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