Investigation of Bacteriophage Larva β-clamp Protein:Protein Interactions

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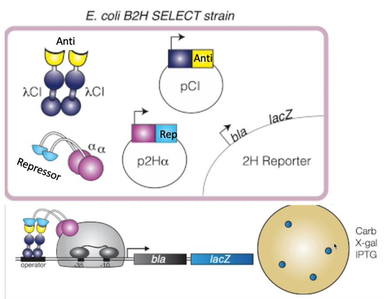
INTRODUCTION / GOALS

DNA is an essential part of any living cell and requires very carefully coordinated repair and replicative processes that involve the work of multiple proteins. In bacteria and bacteriophages, the beta sliding clamp is a homodimer, ring-shaped protein that encircles DNA and serves as a mobile hub for other proteins to bind and carry out DNA maintenance. Protein interaction partners bind the β-clamp through a conserved clamp binding sequence to allow them to transiently associate with DNA, and multiple reports have shown that the bacterial clamp binds proteins involved in both DNA replication and repair. Bacteriophage Larva is unusual in that it contains a gene that encodes for a beta clamp but lacks genes that would produce proteins that typically interact with the clamp such as a clamp loader, a DNA ligase, and a replicative DNA polymerase. We hypothesize that the viral beta clamp interacts with bacterial host proteins during bacteriophage genome replication. To test this hypothesis, we are using a bacterial two-hybrid assay to observe which proteins from both bacteriophage Larva and *Mycobacterium smegmatis* interact with the Larva clamp protein. The goal of this study is to generate a protein interaction map that helps us better understand how a viral clamp protein can enhance viral DNA replication inside the host cell.

METHODS

The bacterial two-hybrid assay is a method of screening for interactions between a protein of interest (bait) and one, or many, possible interacting partners (prey). It utilizes *E. coli* cells that have been transformed with two different plasmids named p2Hα and pCI. p2Hα contains the gene of interest, which is the β-clamp of bacteriophage Larva in this study. The pCI plasmid contains a single gene, or a library of genes, to test for interactions with β-clamp. In this study, pCI either contains a gene predicted to bind the Larva clamp, or a library of genes from *M. smegmatis*, a known host for bacteriophage Larva. Cells transformed with both plasmids are plated on media supplemented with spectinomycin, kanamycin, and chloramphenicol, and those that have successfully taken up both plasmids will have resistances to these antibiotics and exhibit growth. Cells are also plated on a selection plate supplemented with all the same antibiotics plus carbenicillin. If the bait and prey proteins interact, they will induce the expression of the beta-lactamase (bla) gene and confirm resistance to carbenicillin as illustrated in the figure below.



RESULTS: DIRECT TWO-HYBRID

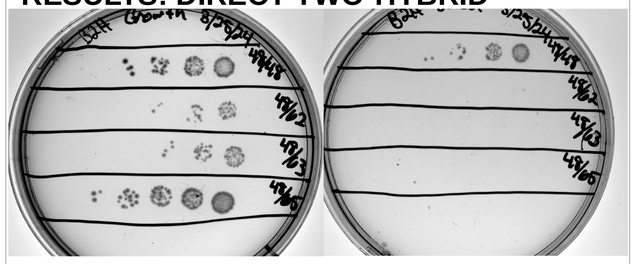


Figure 1. Two-hybrid assay testing the Larva β-clamp for interactions with other Larva proteins. These include two Larva endonucleases (genes 62 and 63) and a helicase (65). Since the β-clamp is a dimer, we placed the clamp gene in both p2Hα and pCI as a positive control. The growth plate is pictured on the left, and the select plate on the right. The results show that while the clamp forms a homodimer that can grow on the select plate (48/48), it does not interact with the protein products of Larva genes 62, 63, and 65.

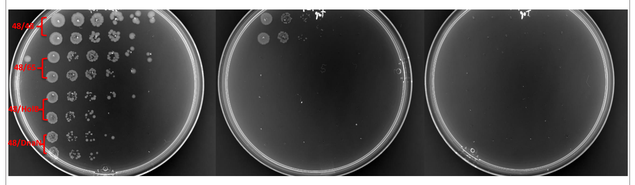


Figure 2. Direct two-hybrid testing for Larva β-clamp interactions with *M. smegmatis* β-clamp (DnaN) and clamp loader (HolB) proteins. This assay includes the growth plate (left), a select plate with 750 ug/mL carbenicillin (center), and a select plate with 1500 ug/mL carbenicillin (right). Assay includes 48/HolB and 48/DnaN combinations, as well as 48/48 and 48/65 as positive and negative controls, respectively. The results again only show growth of the Larva clamp homodimer.

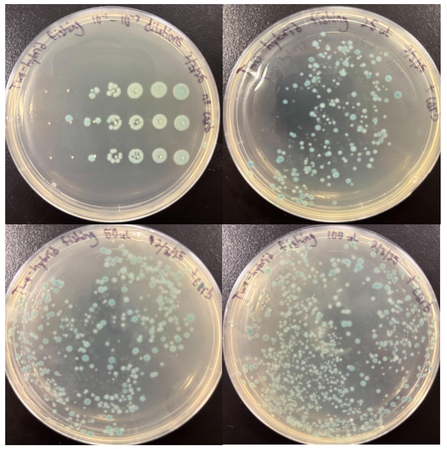


Figure. 3 A two-hybrid "fishing" assay that tests the Larva β-clamp against an entire library of *M. smegmatis* protein fragments. The media in this assay is supplemented with X-gal, which causes colonies to turn blue when a protein:protein interaction occurs. A dilution plate (top left) was made with 10^{-1}–10^{-7} cell dilutions to calculate transformation efficiency. 25 (top right), 50 (bottom left), and 100 (bottom right) uL of transformed cells were plated on growth plates in order to obtain single colonies that could be isolated for subsequent sequencing.

The table on the right shows the *M. smegmatis* gene hits identified from the two-hybrid assay. Individual colonies were isolated and grown in liquid culture, then the plasmids purified and sequenced by Sanger sequencing. The results indicate that the assay has provided non-specific hits; however, the highlighted hits are either hypothetical proteins or proteins predicted to be involved in DNA metabolism that may be legitimate interactions.

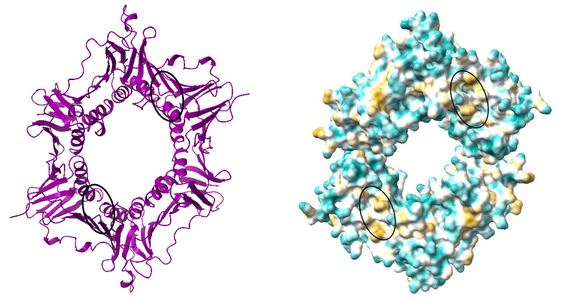


Figure 4. Alphafold model of the Larva β-clamp dimer shown in cartoon (left) and as a surface representation colored by hydrophobicity (right). The sliding clamp interacts with other proteins via a hydrophobic binding pocket between the 2nd and 3rd domains of the clamp monomer. When the monomers form a functional clamp dimer, there are two resulting binding sites which have been circled in this figure.

Figure 5. Alphafold model of a Larva clamp/*M. smegmatis* clamp heterodimer. The Larva clamp monomer is colored in purple, and the *M. smegmatis* clamp monomer is blue. The two monomers are unable to form a closed functional dimer, which agrees with the two-hybrid results.

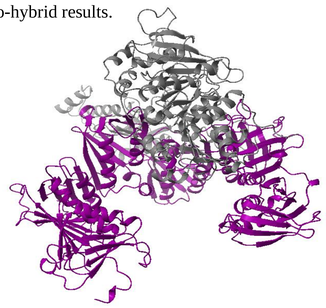


Figure 6. Alphafold model of the Larva β-clamp with the Larva helicase. The clamp is shown in purple, and the helicase is gray. A functional interaction is not predicted to occur, as evidenced by the clamp not forming a closed ring structure in the presence of this helicase. This again agrees with the two-hybrid results that suggest these proteins do not interact.

Sample Sequenced	Hit
1	CaiB/BaiF CoA transferase family protein
2	quinone oxidreductase family protein
3	carotenoid oxygenase family protein
4	hypothetical protein
5	Response regulator
6	nuclear transport factor 2 family protein
7	L-carnitine dehydratase/bile acid-inducible protein F
8	Cobalamin synthesis protein/CobW family GTP-binding protein
9	GntR family transcriptional regulator
10	quinone oxidoreductase
11	Phosphotransferase
12	Hypothetical Protein (different from sample 4)
13	sigma-70 family RNA polymerase sigma factor
14	LLM class flavin-dependent oxidoreductase
15	IS630-like element ISMsm5 family transposase
16	glycoside hydrolase family 65 protein
17	NADH-ubiquinone oxidoreductase-F iron-sulfur binding region domain-containing protein

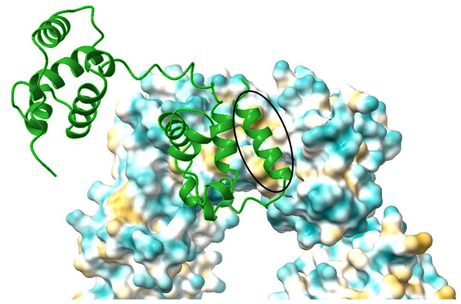


Figure 7. Alphafold model of the Larva β-clamp with the possible transposase (outlined in green) interaction partner from *M. smegmatis*. The model shows an α-helix of the transposase in contact with the hydrophobic binding pocket of the sliding clamp, which has been circled. This model suggests a possible legitimate interaction between these two proteins.

CONCLUSIONS AND FUTURE WORK

Bacteriophage Larva does not possess many genes that code for replicative and repair proteins, but the assays performed have shown that its β-clamp does not interact with the few maintenance proteins that Larva does possess. This phage also does not have a gene for a clamp loader, which is required for attaching the β-clamp to DNA. It was hypothesized that the clamp loader of the host, *Mycobacterium smegmatis*, would interact with the Larva sliding clamp. These assays show that the *M. smegmatis* clamp loader does not interact, and that its own β-clamp can not form a heterodimer with the viral clamp. The two-hybrid fishing experiment was used to flush out possible interaction partners from a library of *M. smegmatis* proteins, with results that indicate most of the protein fragments may be "sticky". Future directions for this study include:

- Evaluating all possible hits from the two-hybrid fishing to find true interacting partners with the Larva β-clamp
- Continue using AlphaFold and ChimeraX to identify the key interacting residues in the hydrophobic binding pockets of β-clamp
- Model interacting proteins to identify conserved binding motifs that facilitate their interaction with the sliding clamp
- Obtain a crystal structure of the Larva β-clamp

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