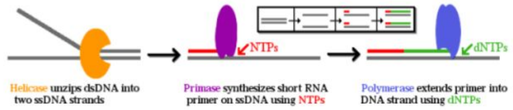
Quantification and Characterization of *Larva64* Primpol Activity

H. Chase Bishop and Dr. Jamie Wallen

Department of Chemistry and Physics at Western Carolina University

Background

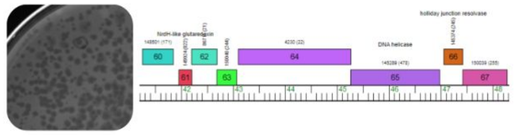
Primase-polymerases, more commonly known as primpols, are enzymes that possess the properties of a primase and polymerase for DNA replication in the same enzyme. DNA replication is an essential biochemical component of all cellular life, and in some viruses. The main aspect that makes something alive is its ability to reproduce, of which replication of DNA to form another organism or to repair itself is of paramount importance. DNA replication usually requires three separate enzymes: **a helicase, a primase, and a polymerase**. Each of these enzymes play an essential role, with the helicase "unzipping" the double stranded DNA, primase forms a small strand of RNA on both pieces of the now unzipped DNA, to which the DNA polymerase will start at the 3'-end of the RNA primers to form complementary DNA. These trifecta of enzymes work together to form two identical strands of double-stranded DNA (1).



While some primpols require a helicase to unzip the DNA, many have the ability to both unzip, prime and polymerize the DNA to undergo DNA synthesis. The **three-for-one property** of primpols is intriguing as it may offer a look into the early origins of DNA replication (1). There have been recent studies that have suggested that certain primpols can function as a helicase as well to undergo strand displacement synthesis (2). Strand displacement synthesis refers to a DNA synthesis that only replicates one side of the DNA strand and releases a piece of single stranded DNA which then becomes double stranded in random fashion. The ability for a singular enzyme to serve multiple roles in DNA synthesis provides impetus for research into primpols.

Primpols are found in a number of bacteriophages. Bacteriophages are viruses that infect solely bacteria, and have the potential as an alternative for antibiotics and in the treatment of antibiotic-resistant bacterial infections. Bacteriophages are the most abundant biological entities on planet Earth, making up an incredibly rich genomic and proteomic landscape to explore. The emerging public health crisis of antibiotic resistant bacteria has resulted in increased interest and research into bacteriophages as clinical therapies (3). Studies of viral genome replication and host-virus interactions can help tailor and adapt bacteriophages for eventual pharmaceutical applications.

Actinophages are typically linear dsDNA viruses that infect bacteria belonging to the Actinobacteria phylum. Mycobacteriophages are of particular interest in the realm of actinophages as they infect certain Mycobacterium hosts. Notable members of the Mycobacterium genus include *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the bacteria responsible for tuberculosis and leprosy (4). Further investigation into mycobacteriophage genomic replication, where primpols are a subject of interest, can potentially help lead to more effective phage treatments against antibiotic resistant infections. Many bacteriophages contain genes annotated as primpols based on domain conservation software; however, when compared to the amount of bacteriophages being discovered and annotated every year, primpols in bacteriophage are largely understudied. Mycobacteriophage Larva was isolated from *Mycobacterium smegmatis* mc^{2}155 in Williamsburg, VA. It is classified as a temperate phage of the Siphoviridae morphotype belonging to cluster K5 (5).



Larva's gene 64 (*Larva64*) encodes a primpol, and is the only encoded DNA polymerase of the phage. Thus, Larva64 is the presumed sole source of Larva's endogenous genetic replication abilities, and prime for study. Previous research at WCU by Nathan Folse in 2020 expressed and purified Larva64and observed *de novo* enzymatic activity by Larva64, as well as protein-protein interactions between Larva64 and *M. smegmatis* proteins via a two-hybrid assay (6). The **enzymatic activity of Larva64 however was not quantified**, rather only shown to be present via gel-based assay and that the enzyme appeared to be Mg^{2+} dependent. The two-hybrid assay shed light on potential interactions between Larva64 and M. *smegmatis* proteins, but did not explore protein-protein interactions within the virus itself.

Moreover, Folse using CRISPRi was able to show that ***Larva64* is necessary** for the survival of phage Larva. However, qualitative data from agarose gel assays and quantitative data from *PicoGreen™* fluorescence assays have shown that the enzyme works at a speed **too slow** to fully replicate the roughly 62 kb viral genome in a timely manner. This indicates other interactions or proteins assist in Larva's genome synthesis.

Goals

- Quantify the DNA synthesis activity of Larva64 via a fluorescence assay
- Identify variables that could potentially increase the speed of *Larva64*
- Determine by what mechanism phage Larva's genome is replicated, and what role *Larva64* plays in that replication given its extremely slow speed.

Methods

General Assay SOP

All assays of *Larva64* activity largely followed the following procedure.

To a 1.5 mL Eppendorf tube set up an assay that contains the following:

- 1 mM dNTPs
- 0.5 micrograms of M13 ssDNA from NEB
- 2.5 uM *Larva64* primpol
- 10 mM $MgCl_2$
- Buffer (1X): 25 mM Tris pH 7.0, 50 mM NaCl, 1 mM DTT, and 100 ug/mL BSA

- Begin reactions with the addition of enzyme. Run in a 37 C water bath
- To quench reactions at desired time points, mix 10 uL of reaction with 10 uL of 0.1M EDTA.
- Quenched solutions are then mixed with *PicoGreen™* or 6X loading dye depending on the assay the samples are being used for.

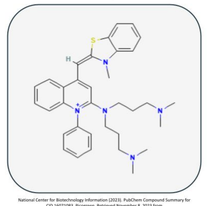
Gel Based Assays

- Quenched and dyed samples are loaded onto a 1% EtBr gel with TAE buffer.
- For samples that require high resolution, a recirculating gel plate is used, running at 20 volts for 999 minutes.
- For samples that require lower resolution and rapid results, standard gels are ran at 100 volts for 1 hour.
- **These assays are QUALITATIVE.**

https://midsci.com/item/B3/Owl-B3-Gel-Electrophoresis/

PicoGreen™ Dye

- PicoGreen™ is an intercalating fluorescent dye which binds to dsDNA.
  - Emission and excitation at 485/528 nm (7)
- Can be used to determine dsDNA concentration, and therefore dsDNA synthesis over time (8).
- CAN'T be used for real time measurements be can be used for quenched assays, as done in this study.
- Fluorescence measured on a SpectraMax iD5 plate reader.



National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 16071083, Picogreen. Retrieved November 8, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Picogreen

Fluorescence Based Assay

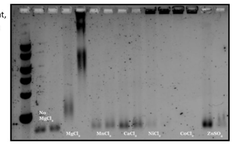
- Quenched samples are mixed with a 5X solution of *PicoGreen™* on a 96 well plate reader plate and given 10 minutes in the plate reader to allow the dye to bind to dsDNA and temperature equilibrate.
- Plate reader settings:
  - Ex, Em: 485, 535 nm (filter engaged)
  - PMT Gain: Low
- A 60 ppb quinine solution (Ex, Em: 349, 461 nm) served as a fluorescence standard between days to account for differing instrument emission intensity.
- **These assays are QUANTITATIVE.**

https://www.gogenlab.com/corning-3686-96-well-half-area-black-flat-bottom-polystyrene-nbs-microplate-25-per-bag-without-lid-nonsterile-cgep-3686/

Characterization of Activity

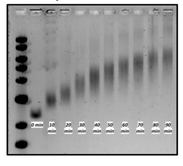
Mg^{2+} Dependence

- Folse in 2020 showed that *Larva64* was Mg^{2+} dependent, unable to substitute Mn^{2+} in lieu of Mg^{2+}.
- To confirm and expand on that result, a gel assay was run with different divalent metal salts replacing $MgCl_2$.
  - $MnCl_2$, $CaCl_2$, $NiCl_2$, $CoCl_2$, $ZnSO_4$.
- No substitute metal salt allowed for activity.



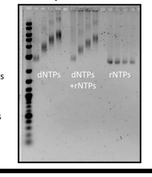
Long Run Timepoints Gel

- To confirm activity of *Larva64* and determine a relative size the dsDNA product formed at a given time, a long run gel based assay was ran.
- Quenched at 10 minute intervals, the gel shows the DNA synthesis activity of the primpol over time.
- Bands above the later time points may indicate strand displacement
- **The product formed after 2 hours is less than 1 kb. Far less than the 62 kb of the viral genome.**



rNTP and dNTP Synthesis

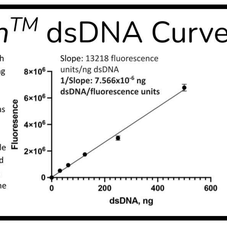
- Given that primpols possess a primase region which utilizes rNTPs, we investigated if *Larva64* would be able to synthesize a double stranded using just rNTPs.
- Standard assay was ran as with rNTPs replacing dNTPs, dNTPs only, and both rNTPs and dNTPs
- rNTPs were unable to form a double stranded product, and the addition of rNTPs neither inhibited or assisted dsDNA synthesis.
- **rNTPs did not speed up synthesis.**



Quantification of Activity

PicoGreen™ dsDNA Curve

- To determine the rate at which dsDNA was being formed using *PicoGreen™*, a M13 dsDNA calibration curve was made.
- *PicoGreen™* concentration was identical to the sample solutions.
- The fluorescence of the sample with no dsDNA was subtracted from the other samples, so as to standardize, which was done for all fluorescence data.



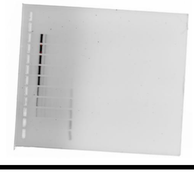
Larva64 Rate and Klenow

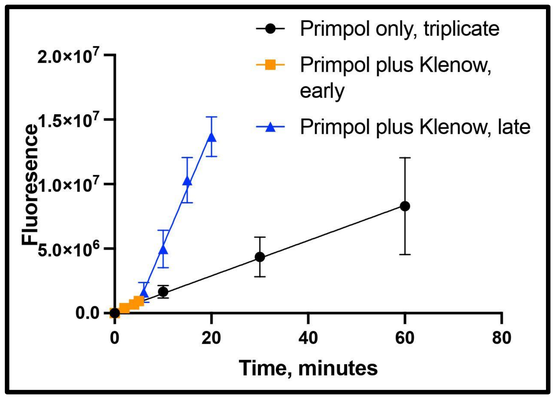
- Multiplying the inverse slope of the dsDNA curve with the slope of the kinetic data (fluorescence/minute), you get a rate with units of **ng dsDNA/minute**.
- Triplicate data of *Larva64* activity using the *PicoGreen™* assay was gathered. The slope of which is 144611 fluorescence units per minute.
  - **Rate: 1.1 ng dsDNA/minute**
- Given *Larva64's* slowness, we decided to see if it could hand off synthesis to a faster bacterial polymerase that is unable to perform *de novo* synthesis: Klenow fragment.
  - Many phages utilize host polymerases for replication.
- The resulting fluorescence chart can be seen in the top right of the poster.

https://www.neb.com/en-us/products/m0210-dna-polymerase-i-large-klenow-fragment

Larva64 and T7 Polymerase

- The addition of a faster polymerase with *Larva64* results in a **short period of dsDNA synthesis by our primpol, followed by the rapid synthesis of dsDNA by the faster protein.**
- This result was seen quantitatively with the bacterial polymerase Klenow, and qualitatively with the phage T7 polymerase.
- ***Larva64* was able to hand off synthesis to both polymerases.**





Discussion and Conclusions

- When Klenow fragment and *Larva64* are mixed in the assay condition, the *Larva64* primpol works until the ~5 minute mark, where it either falls off or **hands off synthesis** to Klenow. Similar qualitative results are seen with T7 polymerase via the gel based assay.
- Since Klenow and T7 polymerase are unable to perform synthesis *de novo* we know that it is *Larva64* which is priming the ssDNA for synthesis and then handing it off.
- This indicates that the *Larva64* may be receiving help from the bacterial host polymerase, and assisting the polymerase by priming for it. A **"team player"** so to speak.
- Since *Larva64* is necessary and is the only encoded polymerase in phage *Larva* we know that it must be playing some part in genome replication.
- Efforts to speed up *Larva64* synthesis using rNTPs and differing salts were unsuccessful.
- The continued slow speed of *Larva64* supports that it is **likely not the sole protein responsible for phage Larva genomic replication.**

Future Work

- Work is underway to utilize a bacterial two-hybrid assay to identify endogenous protein-protein interactions between Larva64 and other genes in the Larva genome that are predicted to interact with DNA.
  - Example: Interaction between Larva primpol and Larva sliding clamp protein.
- These protein-protein interactions could potentially assist in the replication of the Larva genome at a higher rate and shed light on the mechanism of genome replication in phage Larva.
- The peculiar inefficiency of *Larva64* may be due to an uncharacterized novel function of it.
  - Bioinformatic analysis will shed light on primpols in cluster K5, and their potential as a CRISPR-adjacent primase polymerase (CAPP).
- Utilizing non-quneching dsDNA dyes, a real time assay of DNA synthesis could be performed, giving a cleaner and more precise rate.

References

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