Investigating the Putative RecA-Like Recombinase Gene

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INTRODUCTION

Our Biochemistry: Molecular Genetics class has partnered with the Immunology class to investigate the expression of JacoRen57's gene 50.

The bacteriophage JacoRen57 – found in Sioux Center, Iowa (accession: MK279840). JacoRen57's genome has sequenced by Pittsburg SEA-PHAGES Institute and fully annotated by Northwestern College students in 2018. A region between gene 49 and 50 caught our attention as there is a large gap between these genes. Almail et al., investigated if this is a transcription regulatory region for genes 49 and/or 50 (2021). This work demonstrated the region has a regulatory function in the direction of gene 50. Based on comparison genomics, gene 50 is a putative RecA-like recombinase (Almail et al., 2019). This protein has several functions including guiding the recombination of DNA within a gene. RecA-like recombinase allows the virus to evolve into new variants which can improve infection and replication. This is crucial for creating diversity in the genome and DNA repair mechanisms (Galletto and Kowalczykowski, 2007).

To continue examination of gene 50 expression, we are working towards developing antibodies for this protein. To do this, the first step is to create an expression construct (Figure 1), express the protein in bacteria, purify the protein, and then use the purified protein to inoculate mice. This poster describes the construction of the expression vector. This work will provide valuable insight into the expression of gene 50, the RecA-like recombinase.

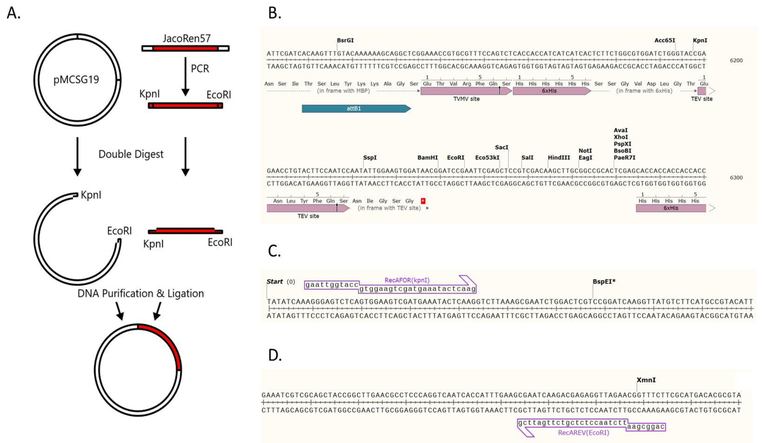

Figure 1. Constructing His-tagged Gene 50. A) A schematic representation of the cloning approach utilizing PCR and restriction enzymes in order to construct pMCSG19 RecA-N-His. B) Region of pMCSG19 into which the coding region of gene 50 will be inserted (between the KpnI and EcoRI sites). C) RecA FOR (KpnI) primer design and template target. D) RecA REV (EcoRI) primer design and template target.

Primer Design

We designed forward and reverse primers to clone the coding region of gene 50 from JacoRen57. The forward primer, RecA FOR (KpnI), consisted of the sequence: GAATT*GGTACC*GTGGAAGTCGATGAAATACTCAAG , and the reverse primer, RecA REV (EcoRI), consisted of the sequence: CAGGC*GAATTC*TAACCTCTCGTCTTGATTCG. Primers were ordered from Integrated DNA Technologies (IDT). The first five nucleotides in each primer were utilized to stabilize the restriction enzyme before cleavage. We included KpnI and EcoRI sites (noted with italics) to enable restriction site cloning into an expression vector, pMCSG19 (Figure 1C and D)(Seiler et al., 2013).

PCR amplification of Gene 50 Coding Region

PCR was carried out in a 50 mL reaction volume as per Promega protocol. A final concentration of 1X Q5 High-Fidelity Master Mix containing an optimized concentration of Q5 polymerase and a final concentration of 200 µM of each dNTP and 2mM Mg^{2+}. 10µM of RecA FOR (KpnI) and 10µM of RecA REV (EcoRI) were combined with varying amounts of high titer JacoRen57 lysate (provided by Dr. Byron Noordewier). The PCR thermocycling program was: 98ºC for 10 seconds, followed by annealing at 58ºC for 20 seconds, and elongating at 72ºC for 1 minute. Thirty cycles were completed. The final extension occurred for 2 minutes. The final PCR product was stored at 4°C. To confirm success of our PCR we ran a 1% percent agarose gel in 1X TAE stained with EtBr. 5 µL of PCR product suspended with a final concentration of 1X loading dye was loaded into each well to confirm successful reaction (Figure 2).

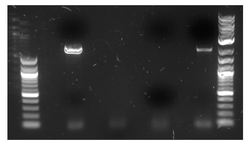

Figure 2. Gene 50 PCR from JacoRen57. Lane 1: 100bp NEB ladder, Lane 2: 1ul high titer JacoRen57 lysate, Lane 3: 5ul high titer JacoRen57 lysate, Lane 4: 10ul high titer JacoRen57 lysate, Lane 5: 15ul high titer JacoRen57 lysate, Lane 6: 1kb NEB ladder**.** The first and last reaction samples contained a product of expected size and were used in subsequent reactions. The faint low molecular weight bands are likely primer dimers.

Following PCR, four separate 50µl double digestions were set up. Each reaction contained 1µg of PCR product, 10 units of KpnI-HF, 10 units of EcoRI-HF (NEB), and a final concentration of 1X CutSmart NEB buffer. Reactions were incubated at 37°C for 1 hour. Subsequent to digestion, the digested insert was purified utilizing spin-based Wizard SV Gel and PCR Clean-Up System (Promega). Confirmation of isolation was done utilizing a 1% Agarose gel (Figure 3).

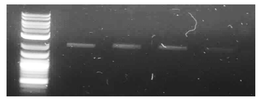

Figure 3. Insert Post-PCR, Digestion, and Purification. Lane 1: 1kb NEB ladder, Lane 2-5: Multiple prepared inserts.

Plasmid Purification

We received the pMCSG19 from DNAsu transformed in *E. coli* (Seiler et al., 2013). The culture was streaked onto LB amp plates (50ug/ml) and incubated overnight at 37°C. Eight colonies were picked and grown overnight in LB amp broth (50ug/mL) for 24 hours. Cultures were pelleted using centrifugation. Plasmid DNA was isolated using the ZymoPURE mini prep kit. The protocol was carried out as per manufacturer's instructions. 25 µL of ZymoPURE Elution buffer was used to elute the plasmid from each column. Successful isolation was confirmed utilizing a 1% agarose gel (Figure 4).

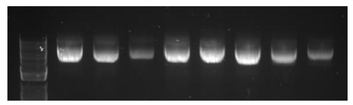

Figure 4. Plasmid Isolation. Multiple colonies were mini prepped in order to have a stock of pMCSG19.

Vector Preparation

Following plasmid isolation 8 separate 50µl double digestions were set up. Each reaction contained 1µg of PCR product, 10 units of KpnI-HF, 10 units of EcoRI-HF (NEB), and a final concentration of 1X CutSmart NEB buffer. Reactions were incubated at 37°C for 1 hour. Subsequent to digestion, the digested insert was purified utilizing spin-based Wizard SV Gel and PCR Clean-Up System (Promega). Confirmation of isolation was done utilizing a 1% Agarose gel (Figure 5).

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Figure 5. Vector Post-Digestion and Purification. Lane 1: 1kb NEB ladder, Lane 2-9: Colony 1-8 miniprep, digestion, and purification products. Colonies 1-5, and 7 were utilized in subsequent steps.

Ligation and Transformation

We carried out a T4 DNA ligase (NEB) using 1-3 molar ratio vector to insert. Ligations were incubated at 14°C for >24 hours. The reactions were then heat inactivated at 65°C for 10 minutes. After reactions were cooled to room temperature, a transformation reaction was carried out. 1 ul of ligation reaction was added to NEB® 5-alpha Competent *E. coli* (High Efficiency) and the transformation was carried out as per manufacturer protocol via the heat shock method.

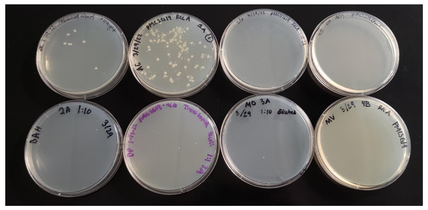

Figure 6. Ligation and Transformation. T4 DNA (NEB) ligase using 1-3 molar ratio vector to insert followed by transformation NEB® 5-alpha Competent *E. coli* (High Efficiency).

FUTURE DIRECTIONS

Protein Expression, Purification, and Injection into Mice

We will express gene 50 in *E. coli* and then utilize Ni^{2+} columns to purify RecA-His protein. The purified protein will be used to generate antibodies in mice. Specifically, we will inject this protein into BALB/c mice for them to raise an immune response against the RecA-like recombinase. These antibodies will be collected from serum and used to do expression studies in *M. smegmatis.*

SOURCES

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