Investigating the –ase:

Understanding the Catalytic Function of *Plasmodium* BEM46-like protein (PBLP)

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Background

What is Malaria?

- Malaria is caused by eukaryotic parasites of the genus *Plasmodium,* which cycle between a vertebrate host and their *Anopheles* mosquito vector.
- Deadliest parasitic disease in the world; symptoms include fatigue, vomiting, anemia, fever, and diarrhea [2].
- *Plasmodium* parasites undergo extensive developmental changes in the host liver, producing infectious merozoites that cause symptomatic disease (Figure 1)[3].

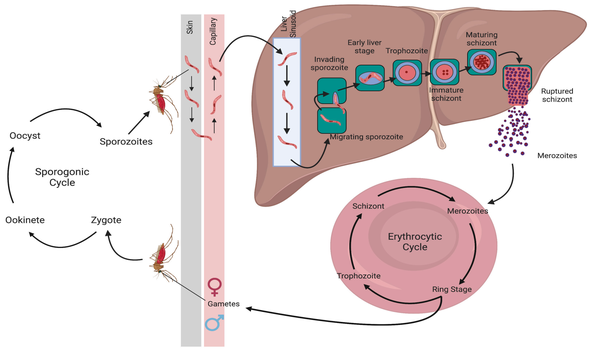


Figure 1. The *Plasmodium* life cycle begins when an infected Anopheline mosquito deposits sporozoites into the mammalian host's skin during a blood meal. These sporozoites enter the bloodstream and reach the liver, where they invade hepatocytes and differentiate into liver-stage parasites. Liver-stage parasites undergo atypical mitotic division, forming a schizont that will release tens of thousands of blood-stage parasites (merozoites). Merozoites will complete their replication cycle in infected erythrocytes, resulting in malaria pathology and symptomatic disease. A small percentage of merozoites develop into gametes, which can be taken up by mosquitoes during subsequent blood meals to reinitiate the infectious lifecycle. Image made using BioRender.

What is PBLP?

- A unique α/β- hydrolase that it is expressed during all stages of the malaria lifecycle [1].
- Membrane-localized during both liver- and blood-stages of the malaria cycle; plays a role in modulating invasive-stage morphogenesis [1].
- Hypothesized catalytic domain consists of three amino acid residues: Serine at the 153rd codon (S153), Aspartic acid at the 229th codon (D229), and Histidine at the 258th codon (H258).
  - **Objective:** Introduce mutations into the catalytic domain of PBLP to discern its role in parasite invasive-stage membrane morphogenesis.

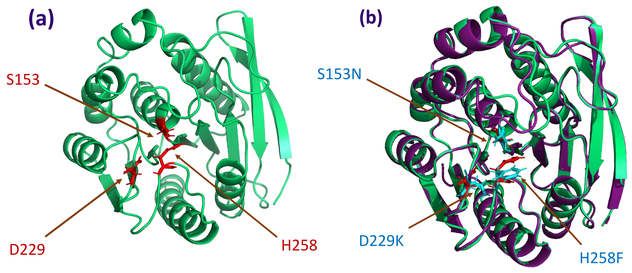
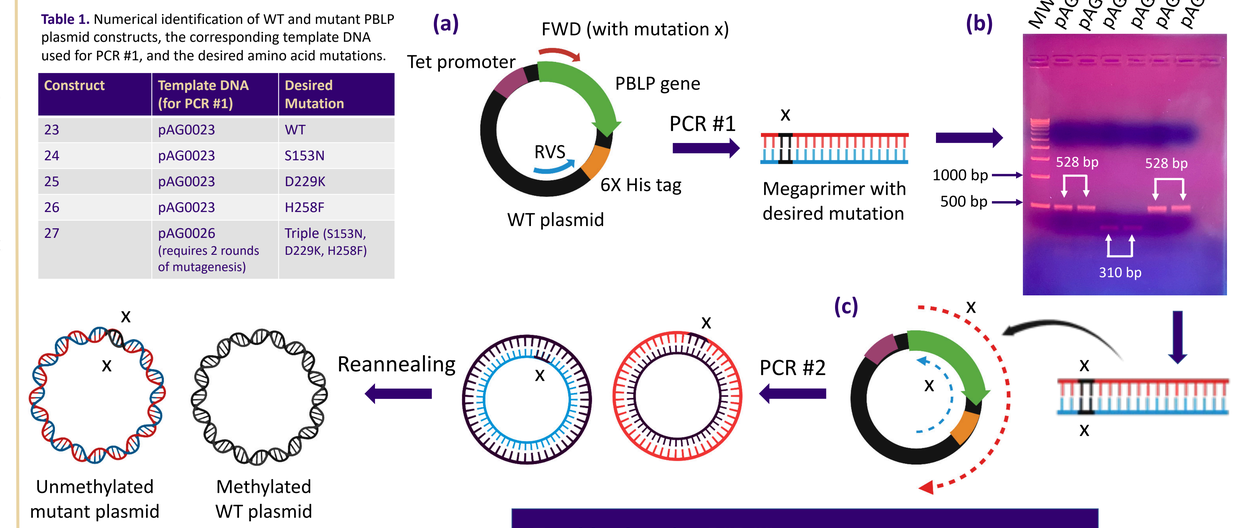


Figure 2. 3D rendering (I-TASSER) of (a) wildtype PBLP (no transmembrane domain) with the putative active site indicated and (b) the desired PBLP triple mutant (purple) with the proposed active site mutations, aligned with the wildtype PBLP (green).

Experimental Road Map

Table 1. Numerical identification of WT and mutant PBLP plasmid constructs, the corresponding template DNA used for PCR #1, and the desired amino acid mutations.

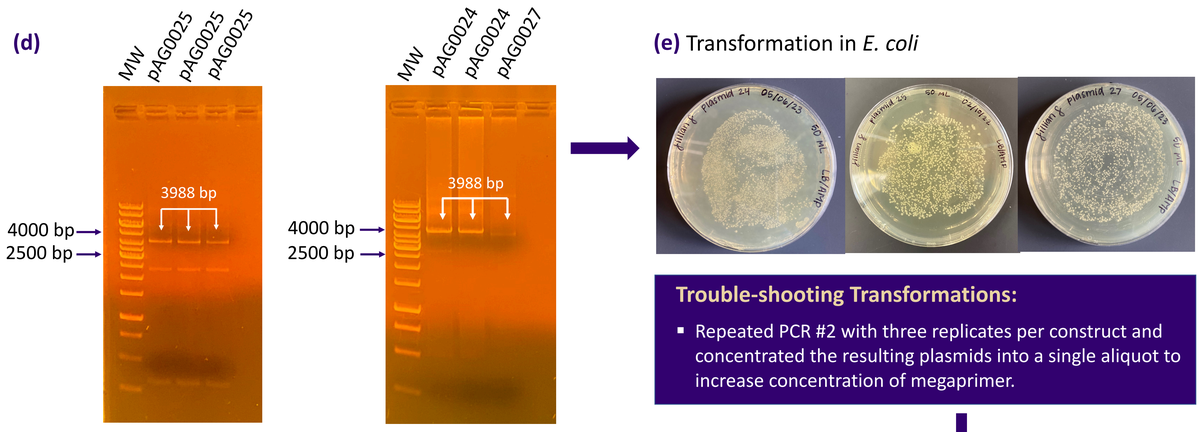
Construct	Template DNA (for PCR #1)	Desired Mutation
23	pAG0023	WT
24	pAG0023	S153N
25	pAG0023	D229K
26	pAG0023	H258F
27	pAG0026 (requires 2 rounds of mutagenesis)	Triple (S153N, D229K, H258F)



Trouble-shooting PCR #2:

- Increasing the megaprimer concentration promotes primer annealing to template DNA.
- Adding DMSO prevents primer dimers and promotes extension.

*Dpn*I Digest
+ Gel Electrophoresis



Trouble-shooting Transformations:

- Repeated PCR #2 with three replicates per construct and concentrated the resulting plasmids into a single aliquot to increase concentration of megaprimer.

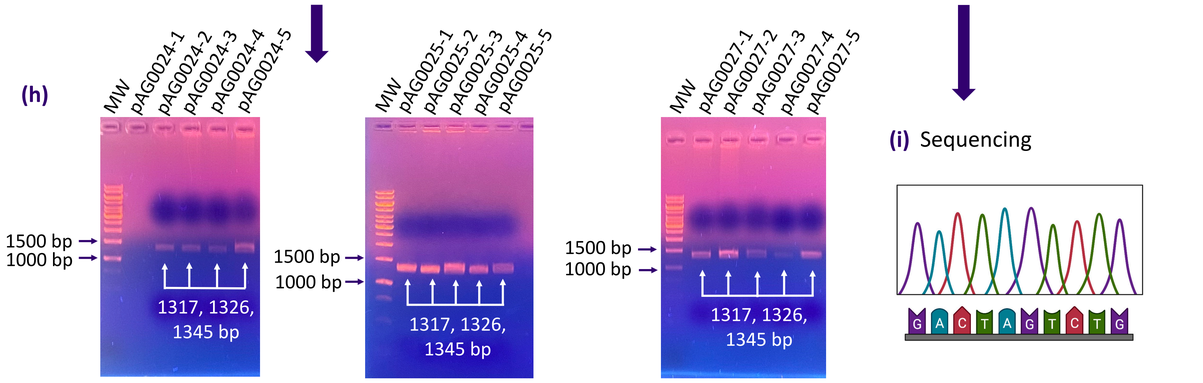


Figure 3. Experimental design to introduce mutations into the wildtype PBLP expression plasmid and disrupt the proposed active site. (a) PCR #1 produced dsDNA megaprimers containing the desired mutations for constructs 24, 25, and 27 using the wildtype PBLP expression plasmid as the template DNA. (b) Gel electrophoresis of PCR #1 to amplify the mutant PBLP expression plasmids using megaprimers with the desired mutations. All three megaprimers were successfully amplified (megaprimers for pAG0024 and pAG0027 are 538 bp, pAG0025 megaprimer is 310 bp). The megaprimers were isolated from the gel using standard PCR clean-up protocols. (c) PCR #2 used the mutant megaprimers to generate whole mutant PBLP expression plasmids. Each reaction used the same template DNA as in PCR #1 and their respective dsDNA megaprimers served as both the forward and reverse primers for PCR #2. Megaprimers denature in the thermocycler and bind to the template DNA where they proceed to extend and form a ssDNA mutant plasmid. The complement ssDNA mutant plasmids anneal to one another and form a whole dsDNA mutant plasmid. (d) Gel electrophoresis from PCR #2 to amplify the whole mutant plasmids with the desired mutations. Mutant construct 25 was the first plasmid to be successfully amplified (3988 bp) and PCR #2 was repeated for mutant constructs 24 and 27, which were also successful. Aliquots for each construct were then treated with *Dpn*I and incubated at 37°C for one hour to eliminate methylated DNA (wildtype plasmids), leaving the mutant constructs intact. (e) Transformation of mutant constructs 24, 25, and 27 into *Escherichia coli,* which were plated on LB plates with 100 ug/mL ampicillin and incubated overnight at 37 °C. Colony formation was seen for each transformed construct and five colonies were selected for plasmid extraction. (f) Overnight LB/Amp (100 ug/mL) cultures of the transformed *E. coli (*pAG0024, pAG0025, and pAG0027) were prepared for subsequent plasmid extraction and sequencing using standard protocols. (g) A diagnostic restriction digest of the mutant constructs was performed using *Dra*I to cut the plasmids into three fragments (1317, 1326, and 1345 bp) and confirm the mutations were introduced. (h) Gel electrophoresis of the *Dra*I restriction digests of purified mutant plasmids. Mutant construct 25 was the first plasmid to be successfully amplified and two samples were selected for sequencing. (i) Standard sequencing reactions were prepared to confirm that the correct mutations (S153N, D229K, and H258F) were introduced into the PBLP expression plasmids. Image made in BioRender.

Conclusions & Significance

- PBLP mutant panel was successfully cloned using MegaWHOP PCR and efforts are underway to complete the triple mutant (pAG0027).
- Mutant PBLP expression plasmids will be used for future biochemical assays to assess protein function *in vitro.*
  - Biochemical studies will further classify the type of –ase by identifying the substrate for the PBLP active site.
- PBLP may serve as an effective anti-malarial drug target, given PBLP is continuously expressed during liver-stage development and has been shown to be important for infectivity [1].

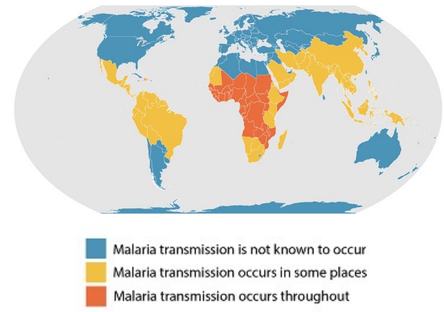


Figure 4. Map approximating the areas in the world where malaria transmission occurs [2].

References

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