Examining Charge-dependent Targeting Signals in *Leishmania* Mitochondrial Protein Import

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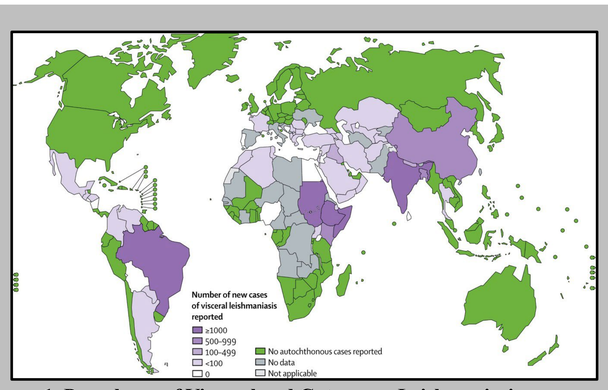
Introduction



- **Figure 1. Prevalence of Visceral and Cutaneous Leishmaniasis**
- **Leishmaniasis is a parasitic disease transmitted to humans through the bite of an infected female sandfly caused by more than 20 different species of *Leishmania* protozoa.**
- **Over 1 billion people are at risk for leishmaniasis, with 700,000 to 1 million new cases annually, mainly in tropical and subtropical regions.**
- **There is no vaccine and drug therapy is unsatisfactory due to toxic side effects and parasitic drug resistance. Development of new therapies against leishmaniasis requires a better understanding of parasite biology, including mitochondrial protein import.**
- **Prior studies of *Leishmania major* (Lm) mitochondrial protein, LmCOX4, show it has an N-terminal positively charged mitochondrial targeting sequence (MTS). This is typically required for protein import into the negatively charged mitochondrial matrix, established by the mitochondrial membrane potential (ΔΨm) of active mitochondria. Further, loss of *Leishmania* ΔΨm that occurs at mammalian temperature does not impair MTS^{LmCOX4}-mediated import.**
- **Surprisingly, replacement of the positively charged amino acids of the COX4 MTS with neutral amino acids does not disrupt its mitochondrial import activity.**
- **This study looks at another *Leishmania* mitochondrial presequence, MTS-SODA. This MTS is derived from the *Leishmania* mitochondrial SODA protein and, unlike MTS^{LmCOX4}, it requires a positively charged presequence for mitochondrial import. This suggests that MTS^{SODA}-mediated mitochondrial import is also ΔΨm-dependent. The goal of this project, is to use PCR and plasmid gene cloning methodology to generate *Leishmania* MTS-reporter expression plasmids. These will allow us, in future studies, to examine whether MTS^{SODA}-mediated mitochondrial import is impaired at mammalian temperature, where *Leishmania* ΔΨm is diminished.**

Figure 2. Cartoon showing MTS fused to Green Fluorescent Protein (GFP) reporter

Amino acid sequences (single letter code) of *Leishmania donovani* and *Leishmania major* SODA MTSs:

LdSODA WT – MFRRVSMKAATATAPVGFSFLCYHTLPHLRYPA (+ Charge)

LdSODA MUT* – MAAVSMAAATATAPVGFSFLCYHTLPHLAYPA (0 Charge)

LmSODA WT – MFGRAPMKAATATAAVGFSCLCYHTLPHLRYPA (+ Charge)

LmSODA MUT* – MFGAAPMAAATATAAVGFSCLCYHTLPHLAYPA (0 Charge)

*** Mutant is (A) as an uncharged control and should not enter the mitochonria**

Note: R & K indicates the positively charged amino acids, arginine & lysine, respectively. A indicates replacement of R or K with the uncharged amino acid, alanine.

Methods

Polymerase Chain Reaction

Each MTS sequence (encoded by a forward PCR primer) was fused to the N-terminus of Green Fluorescent Protein (GFP), with the C-terminus of GFP encoded by a GFP C-terminus reverse primer. In future studies, the GFP reporters we are generating in this project will allow fluorescent visualization of its localization in live *Leishmania* cells. Hence, a functional MTS will specify a mitochondrial fluorescent distribution pattern that can easily be discriminated from mislocalization to the cell cytoplasm. Each MTS-GFP gene fusion was amplified by PCR, and the expected 0.8kb fragments confirmed by Agarose Gel Electrophoresis.

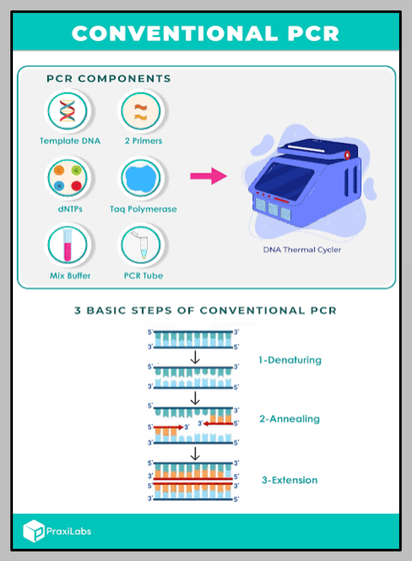


Figure 3. PCR components and steps

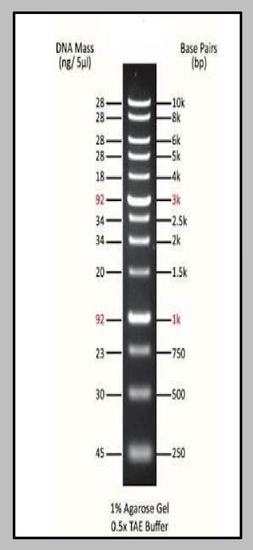


Figure 4. 1kb DNA Ladder

DNA Digest & Gel Purification

After confirmation of each MTS fragment, Pac1 & Xba1 restriction enzymes were used to cut the fragment at each end to create matching sticky overhanging ends. This will allow the PCR Product to insert into the Pac1/Xba1 sites of the pXG plasmid vector for appropriate orientation for correct expression in the *Leishmania* parasite.

The expected 0.8kb Pac1/Xba1 fragments containing MTS-GFP were run on an Agarose Gel, then excised, and purified prior to ligation.

Ligation & Transformation

Insertion of the target MTS-GFP gene into the pXG plasmid vector was done using T4 DNA ligase.

The ligation was then transformed into competent *E. coli*, followed by plating on carbenicillin plates, to select for *E. coli* clones (colonies) containing the intact pXG-MTS-GFP plasmids. Individual colonies were each isolated and expanded into 2 ml volumes for plasmid mini-preps. Plasmids were purified by alkaline lysis, using a Qiagen QIAprep Spin Miniprep Kit. Presence of the appropriate MTS-GFP insert within the pXG vector was confirmed by restriction digest/agarose gel analysis.

Results

As shown in Figure 5, 0.8 kb MTS^{SODA}-GFP (WT) and an uncharged variant of MTS^{SODA} , $MTS^{SODAR4A}$-GFP, were successfully generated by PCR.

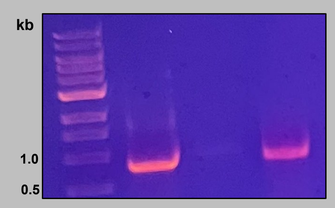


Figure 5

Following ligation of 0.8kb fragments into pXG and transformation , over 100 carbenicillin-resistant colonies were obtained on each plate (Figure 6). This indicates that the ligation reactions were successful.

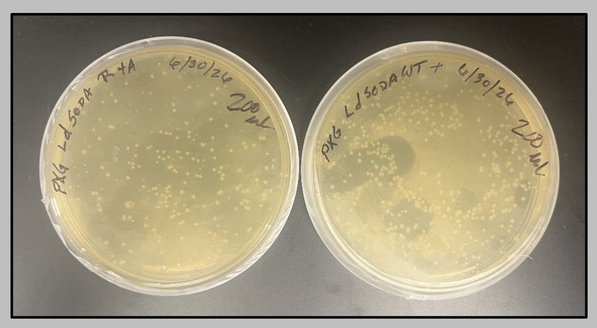


Figure 6

Digestion of the plasmids with restriction enzymes PacI and XbaI yielded the expected fragment sizes according to the plasmid map (Figure 7). These correspond to the MTS-GFP (0.8kb) and the pXG vector backbone (~ 7kb) fragments, observed in Figure 8.

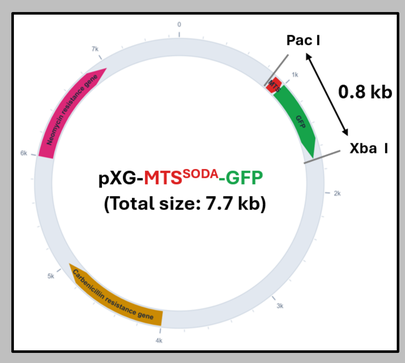


Figure 7

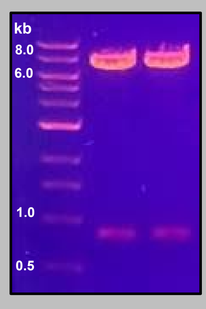


Figure 8

Conclusion

We have successfully generated pXG expression vectors that will allow us to examine whether the charge-dependent mitochondrial targeting sequence of LdSODA is functional under physiologically relevant conditions of ΔΨm depletion resulting from exposure of the parasite to mammalian temperature.

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