# Preliminary Studies of the Biological Activity of the Anaplasmal Appendage Associated Protein

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**ABSTRACT**

The objective of this project is to identify the binding partner of the anaplasmal appendage associated protein (AAAP) produced by *Anaplasma marginale*, the causative agent of bovine anaplasmosis. AAAP is expressed by *A. marginale* that infect bovine erythrocytes and is found in F-actin laden macromolecular complexes known as 'inclusion appendages' in the host cell cytoplasm (Figure 1). However, the role of AAAP in inclusion appendage formation is not understood. The working hypothesis for this project is that AAAP binds to eukaryotic actin binding proteins associated with the junctional complexes in mature erythrocytes. Our approach to testing this hypothesis is to over-express and purify rAAAP and to incubate the protein with lysed erythrocytes followed by affinity chromatography specific for polyhistidine and glutathione S-transferase tags incorporated into rAAAP. Proteins bound to the rAAAP will be isolated through SDS-PAGE, compared to tag and erythrocyte controls, and identified by mass spectrometry. Data gathered from the experiments may show spectrin, tropomyosin, and/or another cross-linking protein from the junctional complex binding to AAAP. Future work will involve unraveling the mechanisms through which AAAP crosses the parasitophorous vacuole membrane, which is also of erythrocyte origin, to become incorporated into inclusion appendages.

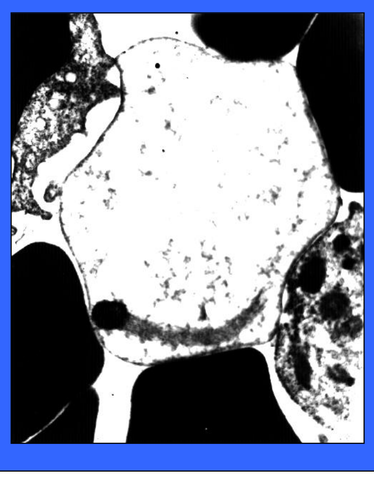

**Figure 1.** This transmission electron micrograph shows the association of F-actin with *A. marginale* to form an inclusion appendage in an erythrocyte (from Stich *et al.*, 1997).

**METHODS**

Determination of AAAP sequences from four distinct *A. marginale* strains are described elsewhere (Stich *et al.*, 2004). The open reading frame sequence encoding AAAP and expressed by the Illinois strain of *A. marginale* was optimized for expression in *Escherichia coli* and this sequence was synthesized and inserted into the expression vector pGS-21a (Figure 2). Two clones of *Escherichia coli* (BL21) transformed with pGS-21a/ILAAAP were selected for expression of rILAAP. Overexpression was induced under two different temperatures and two concentrations of isopropyl-beta-D-thiogalactopyranoside (IPTG) to determine optimal conditions for production of soluble rILAAAP, which was determined with SDS-PAGE. Following induction under the optimized conditions, spin columns were used for affinity chromatography with Ni+ or glutathione laden agarose beads to respectively bind to polyhistidine or glutathione-S-transferase tags within the fusion protein. The purified fusion protein will serve as 'bait' to bind to unknown 'prey' proteins in bovine erythrocytes, and the protein complex will be immobilized with affinity chromatography for the GST or His tags fused to the rILAAAP. After collecting the protein and its binding partner, the components will be resolved with SDS-PAGE and identified using mass spectrometry.

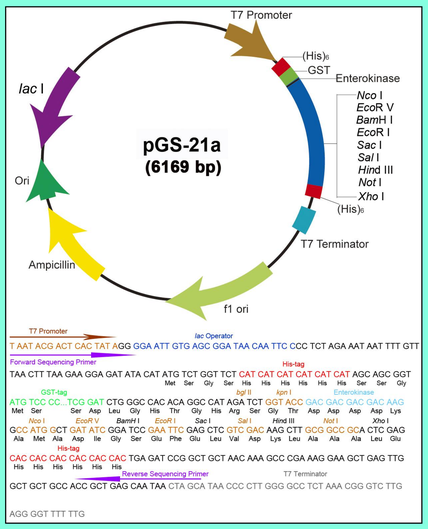


**Figure 2.** This diagram represents the vector transformed into *E. coli* (BL21) for rAAAP expression.

**DATA/RESULTS**

After optimizing the induction time for each of the two clones, inductions under different incubation temperatures, 37°C and room temperature (approximately 25°C), and different IPTG concentrations, 0.5 mM and 1.0 mM, were performed and protein expression was analyzed by SDS-PAGE (Figure 3). The first clone showed optimal expression at one hour post-induction, incubated at 37°C, and induced with 0.5 mM IPTG. The second clone showed the most protein expression at three hours post-induction, incubated at 37°C, and induced with 1.0 mM IPTG. Of the two clones, the first was selected for purification with both Ni+ and glutathione spin columns. The Ni+ spin column provided a higher, though less pure, protein yield overall (Figure 4).

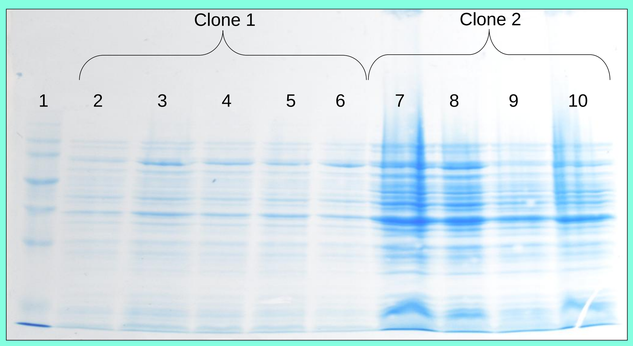


**Figure 3.** This SDS-PAGE gel shows a side-by-side comparison of protein expression in the pellets of the two clones induced under different conditions. Lane 1. Novagen Perfect Protein Marker 10-225 kDa, Lane 2. Pre-induction, Lane 3. One hour post-induction at 37°C with 0.5 mM IPTG, Lane 4. One hour post-induction at 37°C with 1.0 mM IPTG, Lane 5. One hour post-induction at room temperature with 0.5 mM IPTG, Lane 6. Two hours post-induction (no 1 hour post-induction sample collected) at room temperature with 1.0 mM IPTG, Lane 7. Three hours post-induction at 37°C with 0.5 mM IPTG, Lane 8. Three hours post-induction at 37°C with 1.0 mM IPTG, Lane 9. Three hours post-induction at room temperature with 0.5 mM IPTG, Lane 10. Four hours post-induction (no three hour post-induction sample collected) at room temperature with 1.0 mM IPTG.

**REFERENCES**

Stich, R. W., Kocan, K. M., Damian, R. T., and M. Fechheimer. 1997. Inclusion appendages associated with the intraerythrocytic rickettsial parasite *Anaplasma marginale* are composed of bundled actin filaments. Protoplasma. 199:93–98.

Stich, R.W., Olah, G.A., Brayton, K.A., Brown, W.C., Fechheimer, M., Green-Church, K., Jittapalapong, S., Kocan, K.M., McGuire, T.C., Rurangirwa, F.R., Palmer, G.H., 2004, Identification of a novel *Anaplasma marginale* appendage-associated protein that localizes with actin filaments during intraerythrocytic infection. *Infection and Immunity* 72: 7257-7264.

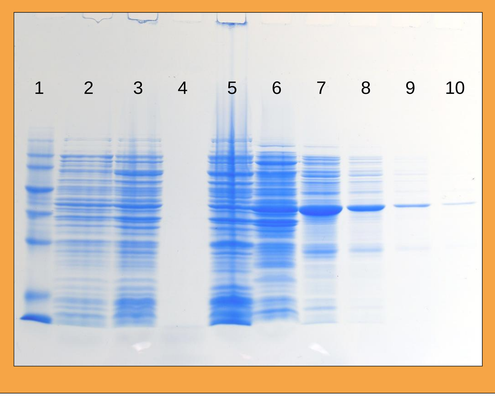


**Figure 4.** The above SDS-PAGE gel shows the results of purification of clone 1 with a spin column using the Histidine tags integrated into the fusion protein. Lane 1. Novagen Perfect Protein Marker, 10-225 kDa, Lane 2. Pre-induction pellet, Lane 3. One hour post-induction pellet prior to addition of B-PER to lyse cells, Lane 4. One hour post-induction supernatant prior to addition of B-PER, Lane 5. One hour post-induction pellet after addition of B-PER, Lane 6. One hour post-induction supernatant after addition of B-PER, Lane 7. First elution from spin column, Lane 8. Second elution, Lane 9. Third elution, Lane 10. Fourth elution.


**Figure 5.** The above SDS-PAGE gel shows the results of purification of clone 1 with a spin column using the GST tag integrated into the expression vector. Lane 1. Novagen Perfect Protein Marker, 10-225 kDa, Lane 2. Pre-induction pellet, Lane 3. One hour post-induction pellet prior to addition of B-PER to lyse cells, Lane 4. One hour post-induction supernatant prior to addition of B-PER, Lane 5. One hour post-induction pellet after addition of B-PER, Lane 6. One hour post-induction supernatant after addition of B-PER, Lane 7. First elution from spin column, Lane 8. Second elution, Lane 9. Third elution, Lane 10. Fourth elution.

**FUTURE STUDIES**

After purification of rILAAAP, the protein will be used to identify other proteins to which it binds through a similar affinity chromatography-based method known as a 'pull-down assay.' rILAAAP will be mixed with lysed bovine erythrocytes and eluted from a Cobalt chelate affinity column to identify any proteins that are bound to the rILAAAP. It is expected that rILAAAP will bind to one or more eukaryotic actin binding proteins associated with the junctional complexes of cortical cytoskeletons in mature erythrocytes. Identifying AAAP's binding partner in the mature erythrocyte is only the first step in determining the protein's biological activity. Further investigation into the process of inclusion body formation and its relevance in host cell infection and pathogenesis needs to be conducted in order to determine if AAAP can be targeted for disease prevention.

**ACKNOWLEDGEMENTS**

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