

## QGEM Results & Discussion

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Project: SAA Expression, ANP Expression, and the Binding Construct

## Western Blotting for SAA secretion

Serum amyloid A (SAA) featured prominently on our list of possible effectors to release at the site of atherosclerotic plaques due to research done at Queen's University by Tam *et al.* (2005). This research showed that treatment of SAA caused macrophages to reverse and prevent esterification of cholesterol, thereby allowing it to be exported out of plaques by high-density lipoprotein (HDL). In order to get the SAA to be picked up by the macrophages we needed to produce large quantities of the molecule, and then secrete it extracellularly. In order to secrete the protein we decided on using the twin-arginine translocase (TAT) system, which can transport fully folded proteins outside the cell (Sargent *et al.*, 2006) and is found in the majority of prokaryotes. We then fused the TAT signal sequence to the front of our SAA sequence in order to produce a construct that should be able produce and secrete the protein, allowing it to be taken up by macrophages.

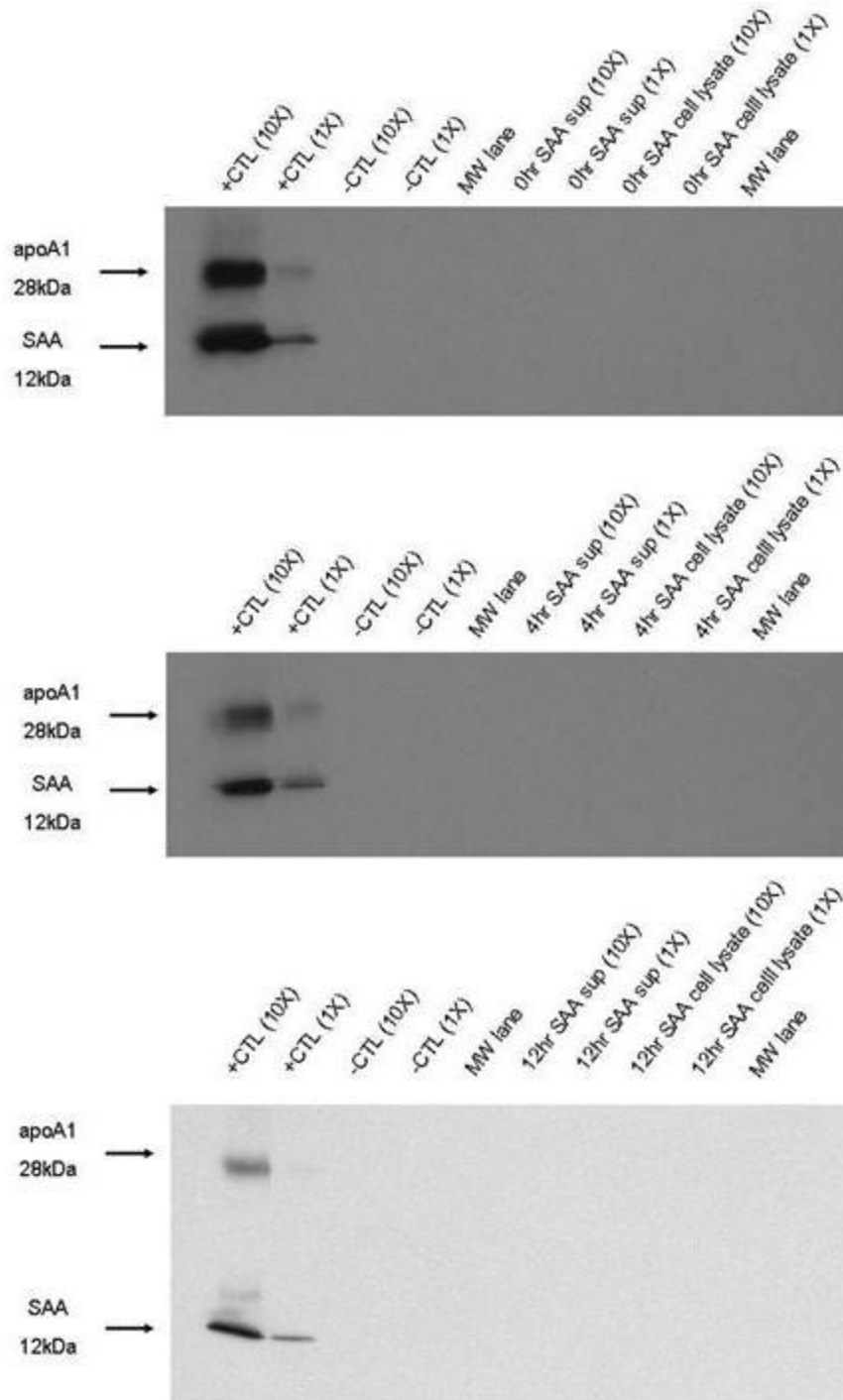


Fig. 1 SAA expression and secretion by *E. coli* cells. Cultures of *E. coli* cells containing either the SAA construct or control plasmid were spun down at 0, 4, and 12 hours after seeding. The culture medium and whole cell lysates were analyzed by SDS-PAGE and Western blot analysis with a polyclonal antibody recognizing whole SAA protein.

Our *E. coli* cells transformed with SAA construct did not appear to express or secrete SAA into the growth medium even after growing for 12 hours in optimum condition. This might be due to defective ligation of the Ptet-RBS fragment to the SAA-encoding gene. We plan to sequence the construct in the future.

Reference:

Benditt, E.P., Hoffman, J.S., and Eriksen, N. SAA, an apoprotein of HDL: its structure and function. *New York Academy of Sciences* **82**:183-189 (1982)

Ancsin, J.B. and Kisilevsky, R. The heparin/heparin sulfate-binding site on Apo-serum amyloid A. *J. Bio. Chem* vol. 274, **11**:7172-7181 (1999)

## Construction of the Binding construct

In our original design of the binding construct, constitutive promoter BBa\_J23119 was used to control the expression of the chimeric protein receptor consisted of the Lpp-OmpA fusion, TEV protease cut sites and ITGA4 fragment. We ordered our construct to be synthesized by Mr. GENE in early June. However, Mr. GENE notified us in August that our construct appeared to be unstable in and toxic to *E. coli* cells. The reason might be that high expression level of Lpp-OmpA might interfere with bacterial physiology, causing severe growth inhibition and reduced viability. (Daugherty *et al*, 1999) Mr. Gene was able to give us a sequence-confirmed PCR product of the synthesized construct. Thus, we decided to replace  $P_{const}$  with  $P_{tet}$  (BBa\_R0040) by PCR. Below is a schematic diagram of the construction process.

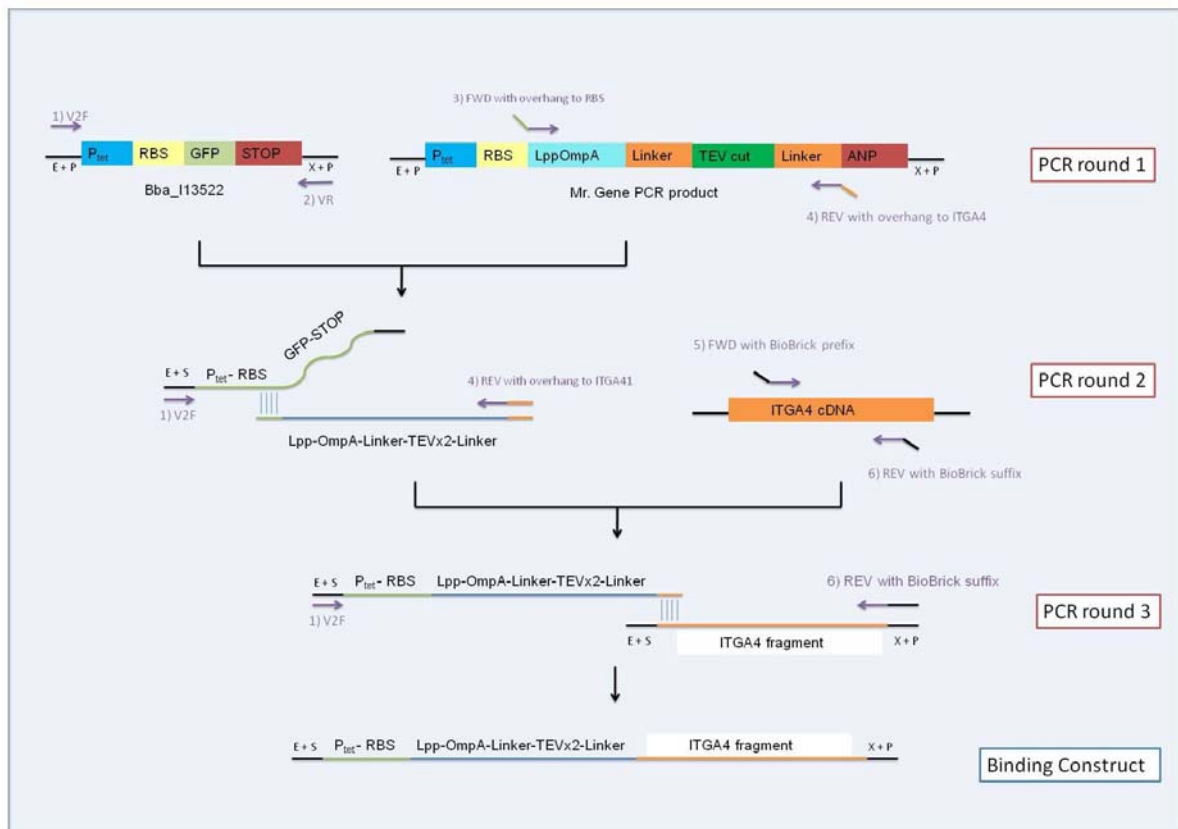


Fig. 2 Flowchart of PCR stitching for constructing the binding construct.

Due to time constraint, we were only able to confirm the stitching of  $P_{tet}$ -RBS fragment to Lpp-OmpA-Linker-TEVx2-Linker fragment (PCR round 2) using Agarose gel electrophoresis. The third round of PCR stitching did not yield expected band. Construction of the binding construct is ongoing.

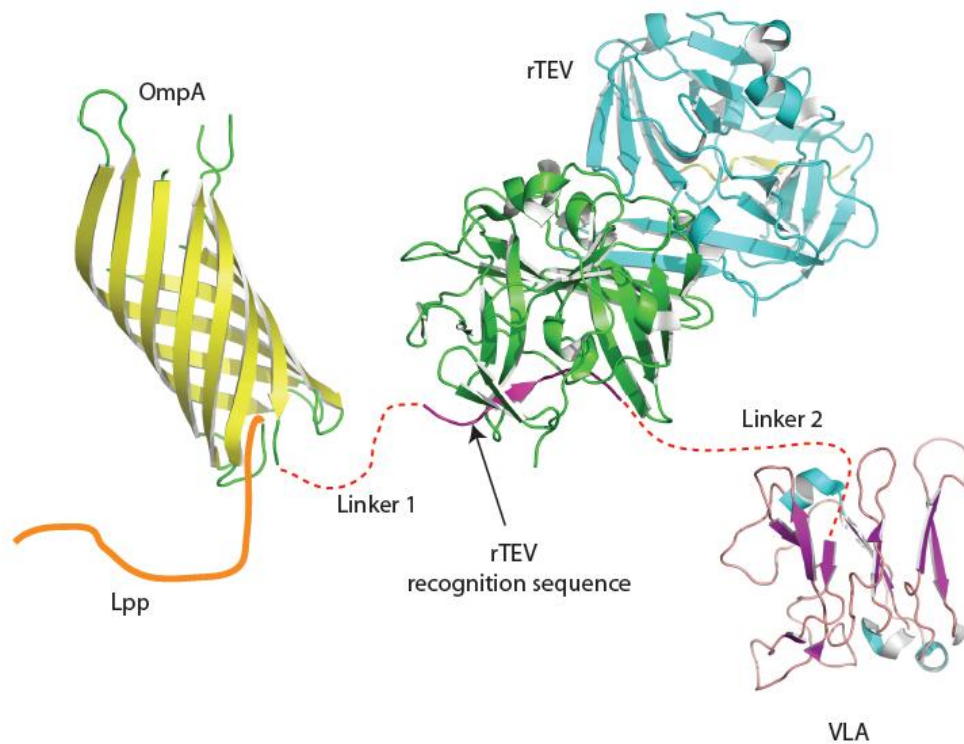


Fig. 3 *In silico* model of the binding construct

Reference:

Daugherty, P.S., Olsen, M.J., Iverson, B.L., and Georgiou, G. Development of an optimized expression system for the screening of antibody libraries displayed on the *Escherichia coli* surface. *Protein Engineering* vol. 12, **7**:613-621 (1999)

Yang, Z., Lui, Q., Wang, Q., and Zhang, Y. Novel bacterial surface display systems based on outer membrane anchoring elements from the marine bacterium *Vibrio anguillarum*. *Am. Soci. Micro.* Vol. 74, **14**: 4359-4365 (2008)

## Future directions

Due to the delay of the synthesis of our binding construct, we were unable to test the presentation of the ITGA-4 chain of the VLA-4 antigen on *E. coli* outer membrane and the binding of the *E. coli* to endothelial cells expressing VCAM-1. In the future, we are planning to pursue this project further in the following areas.

### Construction

1. Complete round 3 of PCR stitching and insert the binding construct into BioBrick backbone.
2. Sequence the binding construct and the SAA construct.

### Endothelial Adhesion Assay

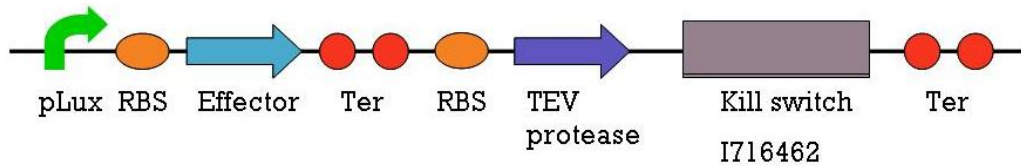
1. Transform the binding construct and constitutive GFP expression construct into *E. coli* cells.
2. Plate C166 murine endothelial cells on a 6-well plate. Incubate overnight.
3. Prepare serial dilutions of the binding *E. coli* and control cells (containing plasmid backbone).
4. Incubate C166 cells with *E. coli* for two hours and then wash wells 3X with PBS.
5. Quantify GFP emission using fluorimeter.
6. Alternative test: ELISA binding assay with recombinant VCAM-1 protein.

### Atrial Natriuretic Peptide

1. Transform *E. coli* cells with ANP construct ( $P_{tet}$ -RBS-Lpp-OmpA-Linker-TEVx2-Linker-ANP) and constitutive GFP expression construct.
2. Use the same protocol as *endothelial adhesion assay* to test the activation of membrane-bound guanylate cyclase (GCA) on endothelial cells by ANP.
3. Harvest the endothelial cells and prepare whole cell lysates.
4. Run SDS-PAGE and Western blotting analysis with antibodies recognizing whole and phosphorylated Vasodilator Stimulated Phosphoprotein (VASP). VASP is phosphorylated by cGMP dependent kinase, which is activated by ANP.

### Inducible effector system

In order to make our *E. coli* cells releasing the effectors at the site of plaque, we plan to put the SAA and HO-1 gene under the control of the  $P_{lux}$  promoter, which will be activated by a threshold concentration of AHL at the site of plaque.



Effector = HO-1, SAA

We plan to transform *E. coli* cells with the effector construct and the binding construct, and conduct the endothelial adhesion assay. We will then analyze the growth medium of the endothelial culture by SDS-PAGE and Western blot analysis to determine the concentration of AHL, SAA, and biliverdin in the medium. We will also determine the expression level of TEV protease, which is located downstream of a double terminator (BBa\_B0015) and RBS (BBa\_B0034) on the effector construct. Expression level of TEV protease directly contributes to the efficiency of detachment from the plaque and termination by the kill switch.