Generation of Limulus Amebocyte Lysate Through Transformation of Mycoplasma

Organism: *M. bovigenitalium*

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Introduction

Nature sometimes brings about organisms that display characteristics formerly thought to only be present in science fiction and an exemplary example of this is the eerie blue blood of *Limulus polyphemus* (American Horseshoe Crab). This copper-based blood contains specialized rudimentary immune cells called amebocytes which produce what is called limulus amoebocyte lysate (LAL). This blood contains pattern recognition receptors (PRRs) which include a myriad of proteins that carry out the functions of the immune system, but the protein complex of focus in this experiment is LAL (Zhou, 2020). When exposed to pathogens, the LAL will utilize many signaling proteins within it and trigger transduction pathways to ultimately defend against unwanted organisms by clotting in the presence of endotoxins produced by many fungi and gram-negative bacteria. During the immune response, the *L. polyphemus*'s blue blood cells will set off what is called a coagulation cascade that is a chain reaction between enzymes and clotting factors to entrap the invading organism due to the lipopolysaccharide or lipoprotein (LPS or LP) coating this invader possesses (Zhou, 2020).

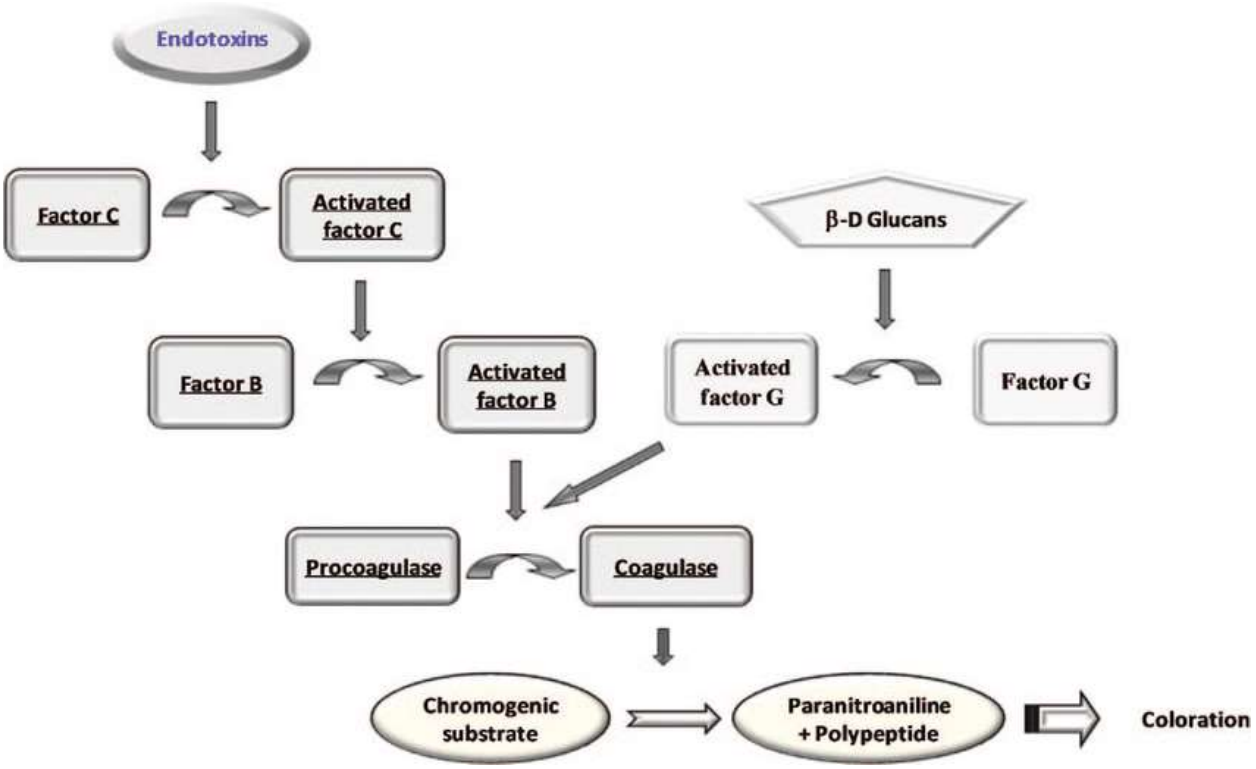

Figure 1. Limulus amebocyte lysate (LAL) coagulation cascade. (Duquenne, 2012).

When the lipopolysaccharides/lipoproteins (LPS/LP) enter the *L. polyphemus*, the cascade begins (Figure 1) with factor C which converts to an activated form that triggers factor B. This factor releases an activation peptide to trigger the pro-clotting enzyme procoagulase, which in turn triggers the clotting enzyme coagulase to trigger the causing a clotting polypeptide and paranitroanaline (a yellow chemical) to form a clot around the LPS/LP (Akbar, 2010; Rietschel, 1994). The clotting component in the blood of *L. polyphemus* has long been used to ensure that tools and treatments in modern medicine are sterile and safe to use and prevent the deaths of many humans during risky procedures such as eye surgeries, brain surgeries, spinal injections, sutures, and vaccines (Akbar, 2010).

Although this blood works miracles and saves many human lives, the *L. polyphemus* pays the price with almost half a million crabs bled yearly with a 10% mortality rate according to a study. This is due to the high stress process on top of aerial exposure for 48 hours and thermal stress (Gisler, P.G et al. 2011; Anderson R.L et al., 2013). This also causes a plethora of sub-lethal behavioral and physiological alterations to the ones that survive which could contribute to a much higher unobserved mortality rate to these bleedings. *L. polyphemus* shows highly decreased hemolymph protein concentration for at least two weeks after the collection of blood. This works in tandem with the depletion of the hemolymph protein hemocyanin which aids in the circulation of oxygen, the primary immune response, cuticle hardening and wound repair (Anderson R.L et al., 2013). The bleeding process of taking 30% of the *L. polyphemus* blood supply even hinders something as basic as movement. As shown in the bar graph below, the bled crabs (grey) exhibit about a 30% decline in movement within the first

Figure 2. L. polyphemus physical strength test post-bleed vs. unbled control. (Anderson, 2013)

week of being bled and it takes around a month to return to normal which opens them up to predation when returned to the ocean (Anderson R.L et al., 2013).

Fortunately, there is a solution to this problem, and it comes through asking the question of how can the proteins necessary for the coagulation cascade be produced without harming *L. polyphemus*? *M. bovigenitalium* is commonly found to cause mastitis and a few other conditions in bovines but is quite helpful and relevant to this experiment due to its' absence of LPS/LP that would normally cause coagulation in the presence of most bacteria (Parker, 2018). Through the utilization of modern techniques such the transformation of plasmids using restriction enzymes, they can be encoded to produce the necessary proteins present in LAL (factors B, C and F) when transcribed within a transformed mycoplasma cell. For transcription to take place, these plasmids must be added to the M. bovigenitalium via the heat shock method. This transformation of the bacteria will cause them to produce the necessary proteins to successfully build the LAL complex.

Methods

Digestion and Recombination of a pUC18 Plasmid Vector

The experiment will begin by obtaining the pUC18 plasmid vector containing an erythromycin resistance gene (selective marker) and adding the restriction enzyme Bbs1 at 37C. Each of these "digested" plasmids will be purified on agarose gel via electrophoresis and extracted into three separate vials to increase efficiency of the DNA + oligonucleotide binding. DNA sequences that have had an oligonucleotide added beforehand will then be combined with the plasmids and will encode each of the elements of the LAL zymogen protein complex (Factor B, C and F). The digested

plasmids will be combined with each of the "sticky end" DNA sequence samples in the separate vials containing buffer solution (100mM Tris-HCl, 10mM EDTA, [pH=8.0]). Each plasmid recombinant will be formed using T4 DNA ligase and ATP at 4C for 12 hours.

Transformation and Isolation of *M. bovigenitalium*

1 µL of each plasmid created to encode for each of the proteins of LAL and erythromycin resistance will then be added to 20 µL of the mycoplasma cells in 50 µL buffer solution (100mM Tris-HCl, 10mM EDTA, [pH=8.0]). This competent cell mixture will then be incubated on ice for 30 min, then heat shocked for 45 sec at 42C, then back on ice for 2 min (Rahimzadeh, 2016). This plasmid/mycoplasma mixture will then be combined with 500 µL of modified Hayflick liquid growth medium containing erythromycin (2.85g heart infusion broth, 90.0 mL distilled water, 20 mL horse serum, 10.0 mL fresh yeast extract, 1.2 mL calf thymus DNA, 1.0 mL thallium acetate, 1.0mL Erythromycin and 0.25 mL benzylpenicillin [pH 7.8]) (Razin, 2012). The transformed bacteria will then be grown at 37C in a shaking incubator for 45 min and then incubated at 37 C overnight (shaking has stopped at this point).

Centrifugation of *M. bovigenitalium* and Isolation of LAL

The transformed mycoplasma will then be treated with 100 µL lysis solution (0.2mol NaOH+0.01mol SDS) centrifugated at 10000 x g @4C for 10 min to sediment the lysed cells. The supernatant will be poured off and centrifugation at 8000 x g @25C for 10 min to sediment larger structures such as organelles. The supernatant that contains the synthetic LAL will be poured off into a vial and stored on ice.

Testing Efficacy of Transformed Mycoplasma LAL

To test the efficacy of the extracted LAL, an experiment will be conducted by comparing reactivity of natural LAL (nLAL) as a control and recombinant LAL against varying concentrations of endotoxin (EU) in solution (50 µL LAL vs. 1.0 EU/mL, 0.75 EU/mL, 0.5 EU/mL 0.1 EU/mL). During each addition, the cuvettes with solution in them will have their amount of yellow color quantified using a UV-VIS Spectrophotometer (405nm) and this will be recorded every 30 seconds for 15 minutes. This experiment will be run in triplicate.

Statistical Methods

Using data from the experiment, the absorbance values (AU405, Y) for both the LAL and the nLAL will be plotted against time (min) in intervals of .5 min to form a standard curve (n=3). A linear regression line will be calculated and the slopes of each of the variables will be used to calculate the Vo values.

The Vo Values (Y) will be plotted on a clustered bar within the graph to represent each concentration (EU/mL) tested. To test to see if the synthetic LAL is comparable to nLAL, an ANOVA test will be run on the Vo values to determine significant differences (n=3); if significant differences are suggested by the ANOVA ($p<0.05$), a two-tailed t-test will be run to compare the nLAL and LAL Vo values at each variable.

Results

Hypothesis: By transforming *Mycoplasma Bovigenitalium* to produce the proteins needed for the LAL coagulation cascade, these extracted proteins will have no significant differences when compared with natural LAL extracted from *Limulus polyphemus*.

Question: Will the Mycoplasma-created LAL factors be as effective as natural LAL factors?

Based on the adjusted (using the blank) absorbance values per 30 seconds, a standard curve will be created from the averages of the triplicate experiments. The Vo values for the nLAL and rLAL are expected to have no significant differences in their slopes. I expect the readings from the spectrometer to get increasingly more yellow (higher absorbance) and have a higher Vo value as the EU/mL of *E. coli* endotoxin increases. I also expect my adjusted Vo values of the recombinant LAL (rLAL) to have no significant difference when compared to natural LAL (nLAL) on the bar graph. Both nLAL and rLAL also shouldn't have significant differences in Vo values when exposed to each endotoxin units (EU) per mL concentration of *E. coli* (10 EU/mL, 0.5 EU/mL, 0.25 EU/mL, 0.125 EU/mL, 0.0625 EU/mL). A possible limitation could be that different proportions of proteins might be produced in the mycoplasma as compared to the *L. polyphemus* blood.

Citations

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