Using a qPCR Technique to Monitor Germination of Bacillus anthracis Spores in Soil

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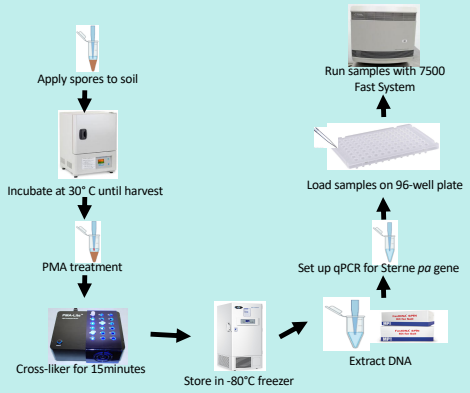
Background

Bacillus anthracis is a Gram-positive, endospore forming, rod-shaped bacterium that causes anthrax, a fatal disease of livestock (primarily cattle, goats, and sheep). This bacterium sporulates under unfavorable growth conditions to produce spores, which are the infectious form of *B. anthracis*. Spores are highly resistant to environmental stressors including extreme temperatures, desiccation, UV and γ-irradiation, oxidation, and harsh chemical treatments[1]. Spores are found worldwide and remain in a dormant, non-reproductive stage for decades to centuries in the soil. However, the introduction of specific nutrient stimuli (germinants) may cause endospores to germinate into vegetative cells[4,6]. It is known that *Bacillus anthracis* efficiently germinates inside a living host, but it is possible that nutrients in the soil may cause germination to occur outside of a living mammalian host.

Objectives

1. The goal of this study is to determine whether *B. anthracis* spores can germinate in soil environments.
2. The Sterne vaccine strain of *B. anthracis* that is attenuated for virulence was utilized, and the PCR amplification target is the protective antigen (*pa*) gene that resides on the pXO1 virulence plasmid[3].
3. 1 X 10^6 *B. anthracis* Sterne spores was applied to 0.5 gm sterile soil for each sample and DNA in the soil was extracted using the FastDNA Spin Kit for Soil.
4. Propidium monoazide (PMA) treatment was applied to eliminate DNA from dead cells or residual DNA in the soil from the real-time PCR results[5].
5. Quantitative polymerase chain reaction (qPCR)[7] will be utilized to quantify DNA from viable *B. anthracis* cells in soil[2].

Experimental Procedure



Results

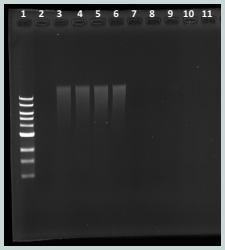


Figure 1. DNA extractions from *B. anthracis* vegetative cells applied soil samples treated with PMA. in various concentrations by FastDNA Spin kit for Soil. The vegetative cell inoculated soils were prepared and divided into two groups, (A) no heat treatment and (B) samples were treated at 65°C for 30 min. PMA of 0, 1.25, 2.5, and 5 mM were added to one sample from each group but the negative controls and incubated in the UV crosslinker. DNA was extracted using FastDNA Spin Kit for Soil. Lane 1: NEB 1Kb Ladders; Lane 2-6 (without heat treatment): cell-free negative control, 0 mM PMA, 1.25 mM PMA, 2.5 mM PMA, 5 mM PMA, respectively; Lane 7-11 (with heat treatment): cell-free negative control, 0 mM PMA, 1.25 mM PMA, 2.5 mM PMA, 5 mM PMA, respectively.

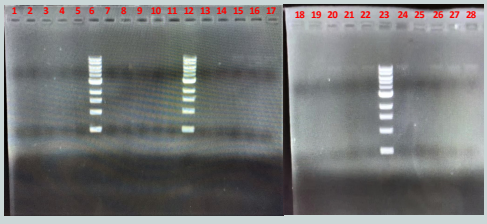


Figure 2. The time course study of *B. anthracis* spore gemination in the soil. The spore soil samples were prepared in triplicate as 1 X 10^6 spores applied to 0.5 gm soil but omitted in duplicate for the negative controls for each time point. The spore inoculated soils were incubated at 30°C and harvested at time points, Day 0,1,2,4, and 8. At harvest, samples were treated with PMA and kept at -80C. At the end of time course, DNA was extracted from all of the samples using FastDNA Spin Kit for Soil. The PCR inhibitor removal kits was applied to the DNA samples. The extracted DNA samples were analyzed on a 0.8 (w/v) % agarose gel.
Lane 1-2: Day 0 control, Lane 3-5: Day 0 samples, Lane 6: NEB 1Kb ladders, Lane 7-8: Day 1 control, Lane 9-11: Day 1 samples, Lane 12: NEB 1Kb ladders, Lane 13-14: Day 2 control, Lane 15-17: Day 2 samples, Lane 18-19: Day 4 control, Lane 20-22: Day 4 samples, Lane 23: NEB 1Kb ladders, Lane 24-25: Day 8 control, Lane 26-28: Day 8 samples.

Summary

1. PCR Inhibitors, such as humid acid, in the soil is removed by washing with 1M Na phosphate, pH 8, before DNA extraction and the DNA samples was cleaned by PCR Inhibitor Removal Kits.
2. It is proved that DNA of ungerminated spores can not be extracted using FastDNA Spin kit for Soil in our preliminary tests.
3. The results of PMA treatment of 1.25-5 mM on soil samples showed no interference on the efficiency of Soil DNA extraction kit.
4. In the time course experiment, DNA electrophoresis showed DNA was extracted in the samples of Day 2-8, but it is yet to determine whether the DNA is from germinated Sterne cells.

Future Directions

1. The qPCR for Sterne *pa* gene will be performed to quantify viable DNA from germinating cells in the soil samples.
2. The time course experiment will be repeated to verify and estimate variability of results.
3. We will test the effect of adding known germinants to the soil and determine relative efficiencies of these treatments on germination.

References

1. **Stewart GC**. October 2015. The exosporium layer of bacterial spores: a connection to the environment and the infected host. *Microbiol Mol Biol Rev* 79(4):438-451
2. **Thompson BM, Lin C-H, Hsieh H-Y, Kremer RJ, Lerch RN, Garrett HE**. September 2010. Evaluation of PCR-based quantification techniques to estimate the abundance of atrazine chlorohydrolase gene *atzA* in rhizosphere soils. *J. Environ Qual* 39:1999-2005.
3. **Bann JG**. November 2012. Anthrax toxin protective antigen-Insights into molecular switching from prepore to pore. *Protein Science* 21:1-12.
4. **Bergman NH, Anderson EC, Swenson, EE, Niemeyer MM, Miyoshi AD, Hanna PC**. August 2006. Transcriptional profiling of the *Bacillus anthracis* life cycle *in vitro* and an implied model for regulation of spore formation. *J Bacteriol* 188(17):6092-6100.
5. **Nguyen LD, Deschaght P, Merlin S, Loywick A, Audebert C, Daele SV, Viscogliosil E, Vaneechoutte M, Delhaes L**. December 2016. Effects of propidium monoazide (PMA) treatment on mycobiome and bacteriome analysis of cystic fibrosis airways during exacerbation. *PLoS ONE* 11(12):1-17
6. **Setlow P**. April 2017. Germination of spores of *Bacillus* species: What we know and do not know. *J Bacteriol* 196(7):1297-1305.
7. **Morrison TB, Weis JJ, Wittwer CT.** June 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Bio Tech* 24:954-962.