2015

Alternate Germinants of C. Difficile, a Leading Hospital Pathogen

Sean Tanzey
University of Nevada, Las Vegas, tanzeys@unlv.nevada.edu

Ernesto Abel-Santos
University of Nevada, Las Vegas, ernesto.abelsantos@unlv.edu

Alternate Germinants of *C. difficile*, a Leading Hospital Pathogen

Sean Tanzey, McNair Scholar, Biochemistry
Ernesto Abel-Santos, Faculty Mentor, Department of Chemistry

Abstract

Clostridium difficile infections (CDI) are the leading nosocomial infections worldwide. Humans are asymptomatic carriers of *C. difficile* spores in the intestinal tract. The process known as germination occurs when otherwise harmless *C. difficile* spores are converted to toxin-producing cells upon recognition of bile salts in humans. This distinctive transition ultimately leads to the onset of disease and recurrent CDI. Germination profiles will be characterized in response to peptidoglycan (PG) fragments isolated from various bacterial species. These specific peptidoglycan fragments contain different amino acid residues that may induce different germination responses. Purification and structural determination of the peptidoglycan fragments will be carried out by HPLC-MS. In this study, *C. difficile* germination will be tested against exhausted media containing cellular debris, as well as with solutions obtained from post-germination assays. This will reveal if germination of *C. difficile* induces other spores to germinate as well. If it is shown that there are alternant germinants of *C. difficile*, further characterization and modeling of *C. difficile* can be made, and further inhibitors can be tested to ensure complete inactivation of spores, ultimately preventing CDI.

Methods

- PG fragments from multiple bacterial species were isolated.
- Germination assays were performed to test the affects of peptidoglycan fragments as germinants on *C. difficile* spores.
- *Bacillus subtilis* str.168 *was* sporulated on supplemented DSM liquid media at 37°C . *C. difficile* was sporulated on supplemented BHI solid media in anaerobic chamber.

Peptidoglycan Isolation

B. subtilis and *E. coli* cells were boiled individually in SDS for 30 minutes, Samples were incubated with mutanolysin for muramidase digestion overnight at 37°C. Debris was removed by centrifugation, and the supernatant was lyophilized. Lyophilized solid was resuspended in 200 µL water and stored at 4°C for later use.

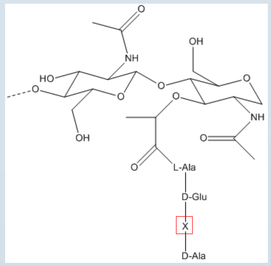
Germination Assay

B. subtilis and *C. difficile* spores were incubated with germinants in germination buffer. Distilled water was used for *B. subtilis* germinants and 0.1M Sodium Phosphate Buffer, pH 6.0 for *C. difficile* germinants. Relative OD_{580} was calculated from OD_{580} measurements taken every minute for 2 hours.

Visualized by Microscopy

Spores were stained with the Shaeffer-Fulton method to visualize germination.

Results



Bacteria	Third AA Residue "X"
E. coli	L-Ala
B. subtilis	m-Dpm

Figure 1: Shortest Peptidoglycan Fragment (disaccharide tripeptide)

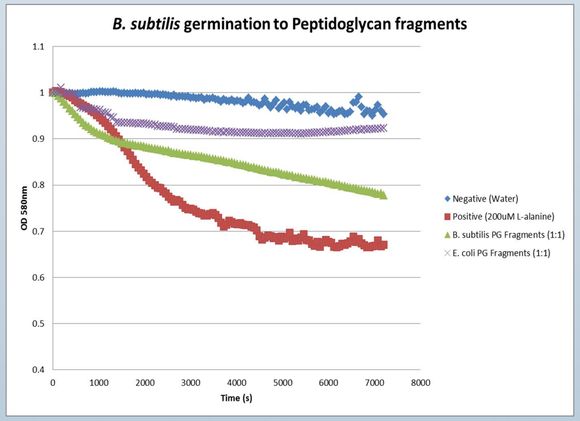


Figure 2: Incubation of *B. subtilis* spores with *B. subtilis* PG fragments displays germination kinetics similar to that of L-alanine, a known germinant.

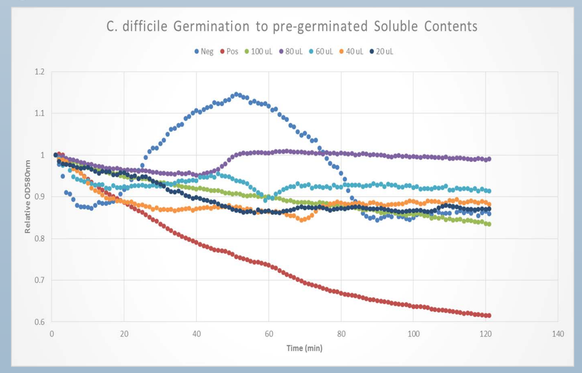


Figure 3: Incubation of *C. difficile* in pro-germination solution has revealed the presence of germinants. Also, DMSO, when added to equal volume of spores, does not provide a stable base line measurement of no-germination.

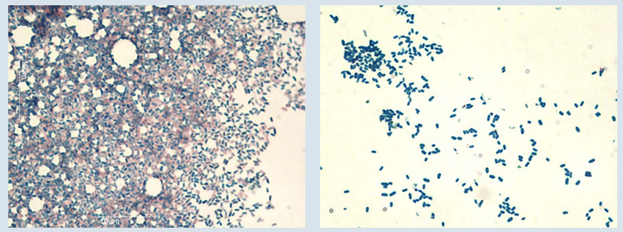


Figure 4: Schaeffer-Fulton stain of Germinated (Green) and non-Germinated *C. difficile* (Red)

Discussion & Future Direction

B. subtilis spores germinated with L-alanine showed 100% germination by a decrease in Relative OD_{580} from 1.0 to 0.6. When *B. subtilis* spores were incubated with unpurified *B. subtilis* PG fragments, a decrease in OD_{580} was detected. This indicated that germination may have taking place, and kinetic analysis of the curve revealed 50% germination. Although this solution contained muropeptides of various lengths, purification can lead to concentrated stocks of the disaccharide tripeptide needed for germination. During *C. difficile* spore germination with a post-germination assay solution, fluctuations in the relative OD_{580} readings were seen. This was most likely due to low concentrations of spores used, leading to inaccurate measurements. Separation of the crude PG fragments will be aided by HPLC for future PG purification, and structure analysis of these fractions will be performed by MS. *C. difficile* germination assays will also be performed with post-germination assay solutions supplemented with known concentrations of taurocholate and glycine for better comparison of kinetics against the appropriate controls. If molecules released during *C. difficile* spore germination can trigger germination of other spores, it could provide reason to search for proteins and the exact molecules responsible for this interaction. An alternate mechanism of germination provides more targets of inhibition for combating CDI.

References

- Abel-Santos, E. (2012). *Bacterial spores: current research and applications*: Caister Academic Press.
- Burke, K. E., & Lamont, J. T. (2014). Clostridium difficile infection: a worldwide disease. *Gut Liver, 8*(1), 1-6. doi:10.5009/gnl.2014.8.1.1
- Mullany, P., & Roberts, A. P. (2010). *Clostridium difficile: methods and protocols* (Vol. 646): Humana Press.
- Shah, I. M., Laaberki, M. H., Popham, D. L., & Dworkin, J. (2008). A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. *Cell, 135*(3), 486-496. doi:10.1016/j.cell.2008.08.039
- Sorg, J. A., & Sonenshein, A. L. (2008). Bile salts and glycine as cogerminants for Clostridium difficile spores. *J Bacteriol, 190*(7), 2505-2512. doi:10.1128/JB.01765-07