UNLV | University Libraries
University of Nevada, Las Vegas

Undergraduate Research Opportunities Program (UROP)

Undergraduate Research Opportunities Program (UROP) 2008

Aug 6th, 9:00 AM - 12:00 PM

Kinetics of Bacillus anthracis and Bacillus cereus spore germination in soil and the C. elegans intestine

MacLean Hall
Davidson College

Ernesto Abel-Santos
University of Nevada Las Vegas, Department of Chemistry, Mentor

Follow this and additional works at: https://digitalscholarship.unlv.edu/cs_urop

Part of the Bacteriology Commons

Repository Citation

Hall, MacLean and Abel-Santos, Ernesto, "Kinetics of Bacillus anthracis and Bacillus cereus spore germination in soil and the C. elegans intestine" (2008). *Undergraduate Research Opportunities Program (UROP)*. 18.
https://digitalscholarship.unlv.edu/cs_urop/2008/aug6/18

This Event is protected by copyright and/or related rights. It has been brought to you by Digital Scholarship@UNLV with permission from the rights-holder(s). You are free to use this Event in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you need to obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/or on the work itself.

This Event has been accepted for inclusion in Undergraduate Research Opportunities Program (UROP) by an authorized administrator of Digital Scholarship@UNLV. For more information, please contact digitalscholarship@unlv.edu.

MacLean Hall
Mentor - Ernesto Abel-Santos

Bacillus anthracis and Bacillus cereus are both described as soil bacteria, but are almost exclusively found as spores within the soil. Soil is generally not a nutrient-rich environment and may lack the amino acids and nucleosides necessary for spore germination and vegetative reproduction. We aim to determine if soil alone can cause germination in these two species in order to produce vegetative cells that can reproduce. In addition, nematodes, decaying meat, maggots, and plant roots will be tested for their ability to cause germination in these species.

Kinetics of *Bacillus anthracis* and *Bacillus cereus* Spore Germination in Soil and the *C. elegans* Intestine

MacLean Hall, Ernesto Abel-Santos

Davidson College, Davidson, North Carolina, 28035

Department of Chemistry, University of Nevada Las Vegas, Las Vegas, Nevada 89154

INTRODUCTION

Historically, *Bacillus anthracis* and *Bacillus cereus* have been described as saprophytic soil bacteria, found almost exclusively as resistant spores in soil devoid of excess nutrients (5, 7, 9). *B. anthracis*, a pathogen specific to mammals, is typically believed to lie dormant as a spore until a suitable host ingests it, allowing germination and infection (1, 7, 9). *B. cereus* is a pathogen of the insect gut and has a close association with the plant rhizosphere, possibly functioning as an important line of defense for its' host. While *B. anthracis* became notorious as the causative agent of anthrax after the 'bioterrorism attacks' of 2001, *B. cereus* is (albeit less-popularly) a known cause of food poisoning and eye infections in humans (7, 9). Until recently, there has been a lack of evidence for vegetative growth in soil, suggesting members of the genus *Bacillus* are obligate symbionts (7, 9). Recent research suggests, however, that there is indeed support for the theory that these two species can proliferate in soil without a host. Vilain *et al.* describes a full life cycle of *B. cereus* in supplemented liquid soil extract and artificial soil microcosms (9). Hanna notes similar results for *B. anthracis*, showing that it can also undergo full life cycles outside of the host in some soil extracts, which may be similar to the life cycle within the host (5).

Despite this breakthrough in understanding *Bacillus* outside of a host, an unexpected host for some members of this genus may be *Caenorhabditis elegans*, a common soil nematode. *C. elegans* normally feeds on *E. coli*, a Gram-negative, non-pathogenic bacterium, but has been observed to eat a wide variety of bacteria (2). Garsin *et al.* show that not all Gram-positive human pathogenic bacteria express similar toxicity towards *C. elegans*, including *B. subtilis* (4). In addition, Anderson *et al.* demonstrated that *C. elegans* will feed on *B. cereus*, but to a reduced degree when compared to other bacteria (2). Furthermore, it is known that nematodes can disperse viable bacteria and simultaneously protect them from harsh environments (2). *C. elegans* is also recognized as able to excrete several different amino acids, including alanine, a known germinant of *B. anthracis* (6). Given this information, it is reasonable to believe that soil nematodes, like *C. elegans* may act as host for members of the *Bacillus* genus and could enable these species to germinate, forming infectious vegetative bacteria.

METHODS

Sieved soil was obtained from Tennessee State University and resuspended in ddH2O equal to 1 ml/g in order to dissolve any organic substances that may be denatured upon autoclaving. The suspension was then filtered using medium fine filter paper (Whatman). The liquid soil extract (LSE) was then filtered through progressively finer pores and ultimately sterile filtered through 0.2 µm filters (Nalgene). The soil was spread thin and autoclaved 5 times. Extract and soil were then checked for sterility by spreading 50 µl of each on LB agar.

Germination was tested in LSE by suspending *B. anthracis* or *B. cereus* spores in extract using an initial OD_{580} of at least 1.000 (corresponds to 10^8 spores) and measuring the absorbance at 1 minute intervals for 60 minutes (Biomate 5, ThermoSpectronic). Germination was not detected over this time period. Previous germination experiments using known germinants (1 mM inosine and 1 mM L-alanine) provided a positive control.

Germination in sterilized soil was tested with (1 ml/g) and without LSE supplement. A minimum of 2×10^8 spores were suspended in either LSE or ddH2O, added to sterile soil and vortexed for 10 seconds. 150 µl of this suspension was transferred to each of 6 wells of a 96-well plate. At 24 hour intervals, including time 0, the contents of each well were sequentially resuspended using 1 ml of ddH2O and transferred to a 1.5 ml eppendorf tube. The contents were immediately vortexed, serially diluted, and spread in triplicate on LB agar using disposable spreaders. The contents were then pasteurized at 75° C for 30 minutes, after which the same procedure was applied as before heating. The plates were then incubated at 37° C for 20 hours, at which point colonies were counted. The 96-well plate containing the other time points was placed in a plastic bag with moist KimWipes and incubated at 37° C between time points. A soil suspension in which all conditions were kept the same, but no spores were added served as the negative control.

C. elegans N2 stock strain was maintained on *E. coli* OP50 at 24° C on NGM (8).

To determine *C. elegans'* preference for cultures of *E. coli* OP50, *B. anthracis*, and *B. cereus*, NGM plates were set up with 50 µl lawns of bacterial cultures on opposing ends of each plate. Certain *Bacillus* cultures were induced to sporulate by allowing them to grow for 7 days at 37° C, while vegetative cells were added on the 6th day. These conditions were also replicated for *E. coli* OP50 as a control, despite the species' inability to form spores. Mixed stages of *C. elegans* were then resuspended in M9 buffer from stock cultures and 20 µl was pipetted medially between the two cultures. Cultures were stored at 24° C and the number of nematodes within each bacterial culture was counted at 24 hour intervals, starting after 12 hours.

Germination in the gut of *C. elegans* was tested by preparing 7 day cultures of *E. coli* OP50, *B. anthracis*, and *B. cereus* on NGM incubated at 37° C. Each of the *Bacillus* species were assumed to form spores by this time. Mixed stages of *C. elegans* were then resuspended in M9 buffer and added to each 7 day plate. In addition, each *Bacillus* culture was supplemented with 500 µl of 100 µg/ml ampicillin. At 24 hour time intervals, excluding the initial time point, five adult L4-stage nematodes were transferred from each 7 day culture plate to BHI agar containing 300 µg/ml erythromycin. The nematodes were washed with a 8.0 µl drop of 50 mM inosine and L-alanine in M9 buffer, then transferred to 1.5 ml eppendorf tubes containing 50 µl 1% Triton in M9. The nematodes were ground using a micropestle and the final volume was adjusted to 100 µL. This solution was spread on PEA agar for *Bacillus* and MacConkey for *E. coli* before and after pasteurization at 75° C for 30 min.

SOIL RESULTS

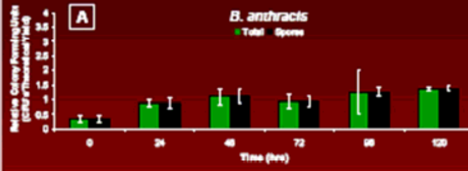
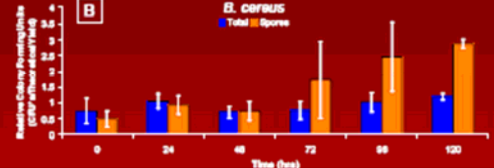


Figure 1. *B. anthracis* and *B. cereus* lack ability to germinate in soil.

B. anthracis (A) and *B. cereus* (B) spores were added to soil supplemented with liquid soil extract and incubated at 37° C for 120 hours. At the specified times, these solutions were resuspended in ddH2O, spread on LB agar (Total), pasteurized (75° C for 30 minutes), and spread again on LB agar (Spores). Both species fail to show a significant reduction in the number of spores present (germination) or 'Total' values ≥ 2 (replication). Data represents average colony forming units divided by the theoretical yield (100 colonies), replicated at least 3 times, and error bars represent the standard deviation. Control = 0 CFU.

Germination was also tested for each species in liquid soil extract and in soil that was supplemented with ddH2O only, but each of these conditions failed to show a significant decrease in the number of spores present, indicating a lack of germination.

Data with greater values of spores than the total count may be due to soil clumping, which can trap bacteria, reducing those counts, while spores may be released during pasteurization, inflating those counts (9).



NEMATODE RESULTS

Nematode Preference

A — 12 Hours

% Preferred -------->	*E. coli* 24 hours (R)	*E. coli* 7 days (R)
E. coli 24 hours (L)	33.33%	60.00%
E. coli 7 days (L)	20.00%	37.50%

B — 36 Hours

% Preferred -------->	*E. coli* 24 hours (R)	*E. coli* 7 days (R)
E. coli 24 hours (L)	60.00%	88.46%
E. coli 7 days (L)	11.04%	44.44%

C — 60 Hours

% Preferred -------->	*E. coli* 24 hours (R)	*E. coli* 7 days (R)
E. coli 24 hours (L)	71.66%	90.09%
E. coli 7 days (L)	9.91%	~50.00%*

D — 12 Hours

% Preferred -------->	*B. anthracis* Vegetative	*B. anthracis* Spores	*B. cereus* Vegetative	*B. cereus* Spores
E. coli 24 hours	66.67%	100.00%	66.67%	66.67%
E. coli 7 days	ND	0.00%	6.67%	0.00%

E — 36 Hours

% Preferred -------->	*B. anthracis* Vegetative	*B. anthracis* Spores	*B. cereus* Vegetative	*B. cereus* Spores
E. coli 24 hours	100.00%	60.00%	60.00%	18.75%
E. coli 7 days	0.00%	25.00%	0.00%	25.00%

F — 60 Hours

% Preferred -------->	*B. anthracis* Vegetative	*B. anthracis* Spores	*B. cereus* Vegetative	*B. cereus* Spores
E. coli 24 hours	6.66%	20.00%	12.77%	0.33%
E. coli 7 days	6.86%	10.71%	1.96%	0.00%

G — 12 Hours

% Preferred -------->	*B. anthracis* Vegetative	*B. cereus* Spores
B. cereus Vegetative	60.00%	100.00%
B. anthracis Spores	100.00%	66.67%

H — 36 Hours

% Preferred -------->	*B. anthracis* Vegetative	*B. cereus* Spores
B. cereus Vegetative	100.00%	100.00%
B. anthracis Spores	100.00%	66.67%

I — 60 Hours

% Preferred -------->	*B. anthracis* Vegetative	*B. cereus* Spores
B. cereus Vegetative	66.67%	40.00%
B. anthracis Spores	100.00%	75.64%

Figure 2. *C. elegans* prefers *E. coli* OP50 over *B. anthracis* and *B. cereus*; *B. anthracis* over *B. cereus* after 60 hours. Mixed stages of *C. elegans* in liquid M9 buffer were added medially to NGM plates bearing two of the above bacterial cultures on opposite sides. *E. coli* OP50, was used as a control (A - C), showing lack of a strong preference between identical culture conditions (designated L and R, for Left and Right), while indicating a preference for the 7 day cultures, used to simulate the effect of sporulation in *Bacillus* species. *Bacillus* spores were incubated for 7 days on NGM plates at 37° C, while vegetative cells were grown in these conditions overnight. Data represents the percentage of nematodes within the bacterial culture highlighted in blue above out of the total number of nematodes within either culture. Only nematodes within each bacterial culture were counted. ND: no worms in either culture. *Surpassed upper limit, represents estimation. Upper limit = 300. n = 1.

Acknowledgments

I would like to thank Monique Akoachere, Norma Ramirez-Ramirez, Denis Nforni, Helen Luu, Andy Phui, and Krystle Tuaño for all of their help and for welcoming me into their lab. Special thanks to Dr. Kudjo Dzantor for providing the soil and Kristin for helping me take pictures with her lab's microscope. I also want to thank Alex Michaud for ideating, Sabrina Schöbbe and Paul Howse for supplies and occasional use of their lab, and all those who attended N.J.S. Funding was provided by the National Science Program (REU 0649267).

NEMATODE RESULTS CONTINUED

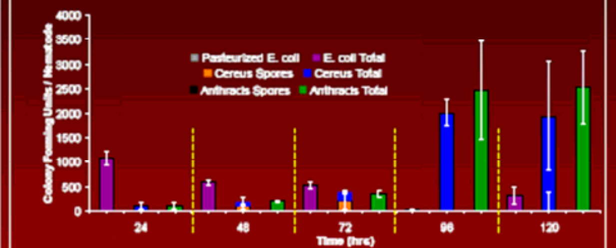


Figure 3. *B. anthracis* and *B. cereus* germinate and replicate in the intestine of *C. elegans*. Mixed stages of *C. elegans* were added to NGM plates containing spores of each *Bacillus* species and *E. coli* OP50 grown under the same conditions. At the specified times, five adult L4-stage *C. elegans* were picked from each plate, transferred to BHI plates containing 300 µg/ml erythromycin, and washed in 8 µl drops of a 50 mM inosine and alanine in M9 buffer. Nematodes were then ground in 1% Triton in M9 buffer to release intestinal bacteria. *Bacillus*-raised nematode gut content was spread on PEA agar to select against *E. coli*, while *E. coli*-raised nematode gut content was spread on MacConkey agar. Performed in triplicate from separate cultures. Data represents average CFU's per nematode. Error bars represent standard deviation.

DISCUSSION

- *B. anthracis* and *B. cereus* do not germinate in the soil received from TSU
  - It has been shown that other types of soil can induce germination; it may be important to understand the properties of these types of soil to protect ourselves and our agriculture industry from proliferating *Bacillus* species
- *C. elegans* prefers *E. coli* to *Bacillus*
  - This could be simply because *C. elegans* has been grown for many generations on *E. coli*. Nematodes isolated from the soil could have very different preferences.
- *C. elegans* prefers *B. anthracis* to *B. cereus*
  - *B. cereus* is very closely related to *B. thuringiensis* and *B. megaterium*, which produces toxins against nematodes
- *B. anthracis* and *B. cereus* germinate in the nematode gut
  - May be the only way the nematodes can digest the spores
  - Potential host and vector for pathogenic *Bacillus*

Future Projects

- Investigate other types of soil using similar techniques and the characteristics any soil that allows for germination
- Use synchronized cultures of *C. elegans* to determine if *B. anthracis* and *B. cereus* have a significant effect on the lifespan of nematodes.
- Isolate the excretion products from liquid cultures of *C. elegans* to determine if this product influences *B. anthracis* and *B. cereus* germination in the gut.

References

1. Akoachere, M., Squires, R. C., Nour, A. M., Angelov, L., Brojatsch, J., Abel-Santos, E. 2007. Identification of an in Vivo Inhibitor of *Bacillus anthracis* spore germination. *The Journal of Biological Chemistry* 282 (16): 12112-12118.
2. Anderson, G. L., Caldwell, K. N., Beuchat, L. R., and Williams, P. L. 2003. Interaction of a free-living nematode, *Caenorhabditis elegans*, with surrogates of foodborne pathogenic bacteria. *Journal of Food Protection* 66 (9): 1543-1549.
3. Darby, C. Interactions with microbial pathogens (September 6, 2005), , ed. The Research Community, WormBook, doi/10.1895/wormbook.1.21.1, http://www.wormbook.org.
4. Garsin, D. A., Sifri, C. D., Mylonakis, E., Qin, X., Singh, K. V., Murray, B. E., Calderwood, S. B., Ausubel, F. M. 2001. A simple model host for identifying Gram-positive virulence factors. *Proceedings of the National Academy of Sciences* 98 (19): 10892-10897.
5. Hanna, P. C. 2004. Towards a complete *B. anthracis* life cycle in soils? Abstract / Unpublished Data.
6. Nicholas, W. L. 1984. *The biology of free-living nematodes*. Clarendon Press, Oxford.
7. Saile, E., Koehler, T. M., 2006. *Bacillus anthracis* multiplication, persistence, and genetic exchange in the rhizosphere of grass plants. *Applied and Environmental Microbiology* 72 (5): 3168-3174.
8. Stiernagle, T. Maintenance of (February 11, 2006), , ed. The Research Community, WormBook, doi/10.1895/wormbook.1.101.1, http://www.wormbook.org.
9. Vilain, S., Luo, Y., Hildreth, M. B., Brözel, V. S. 2006. Analysis of the life cycle of the soil saprophyte *Bacillus cereus* in liquid soil extract and in soil. *Applied and Environmental Microbiology* 72 (7): 4970-4977.