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Literature Review on Mechanisms that Affect Persistence of *Bacillus anthracis* in Soils

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Literature Review on Mechanisms that Affect Persistence of *Bacillus anthracis* in Soils

U.S. Environmental Protection Agency
Cincinnati, Ohio 45268

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Executive Summary

Risk associated with the presence of *B. anthracis* in the environment in the United States, depends on a variety of bioclimatic factors such as soil type, environmental conditions, and ecology, as well as bacterial lifecycle, persistence, and potential to lose (or gain) virulence. Persistence is the ability of an organism to remain viable (e.g., to remain alive) over time under a given environmental condition and medium. The purpose of this study was to determine the current state of scientific knowledge regarding the persistence of *B. anthracis* spores and vegetative cells in soil matrices. Information about *B. anthracis* for this literature review was considered from reports, peer-reviewed journal articles, books, and government publications focusing on publications in the last ten years, but including relevant information dating back to the 1940s from open literature. The agent name and "persistence" or "survival" were used as the primary search terms, but the terms "viability" and "recovery" were also included. This report describes a multifaceted *B. anthracis* lifecycle in combination with genetic, ecologic, and meteorologic factors that have enabled *B. anthracis* spores to persist in the environment.

The vegetative form of *B. anthracis* does not compete well with other soil microorganisms and is often considered an obligate pathogen, unable to reproduce outside of an infected host in nature. Establishment of a persistent cycle from a host, to soil, to a host is the basis for recurrent outbreaks. However, recent work has noted that vegetative cells may persist up to 120 hours in topsoil and an inoculated mixture of spores and vegetative cells on topsoil persisted up to 56 days [1]. The spore possesses numerous protective properties. The outer exosporium layer of the spore tends to be more hydrophobic, which aids the spore in adhering to the surrounding environment.

In addition to the stability of vegetative cells, *B. anthracis* has more recently been found to survive and grow in non-host microenvironments, which contradicts with the obligate mammalian pathogen theory. For example:

- *B. anthracis* was found to propagate in the rhizosphere of a common pasture grass and evidence of plasmid transfer between two strains of *B. anthracis* in the model rhizosphere system was observed[2]. This finding is significant as it provides evidence of metabolically active *B. anthracis* cells in the plant-soil environment. It has been hypothesized that growth of *B. anthracis* within rhizome nodules might be the missing environmental reservoir of *B. anthracis* between natural anthrax outbreaks[3]. However, other studies have found that the presence of the grass does not necessarily increase *B. anthracis*

[1] U.S. EPA, 2014. Environmental persistence of vegetative *Bacillus anthracis* and *Yersinia pestis.* EPA/600/R-14/150.
[2] Saile and Koehler, 2006. *Appl Environ Microbiol* **72** (5): 3168-3174.
[3] Kiel *et al.* 2009. In *Proceedings of SPIE* eds. Fountain, A.W., III and Gardner, P.J. Bellingham, WA

survival or multiplication in the soil, and theorized that the presence of *B. anthracis* in soil may promote establishment of grass at carcass sites, thereby potentially increasing the transmission of *B. anthracis* to grazing hosts by attracting more grazing animals to the site[4].

- *B. anthracis* spores have been found to germinate within common soil amoeba and replicate to the point of lysing the amoeba host. Upon lysis, the vegetative cells will sporulate in a simulated stagnant water/moist soil environment. Research has also found a pX01 plasmid dependence for spore germination within the amoeba, which might explain why a second plasmid associated with infection, pX02, is lacking in multiple natural *B. anthracis* strains – it is not required for proliferation[5].

- Similarly, an interaction between the intestinal tract of earthworms and *B. anthracis* has been shown to be dependent upon the presence of various bacteriophages (viruses that infect bacteria). Bacteriophage infection of *B. anthracis* could create lysogens (the integration of phage nucleic acid into the bacterial chromosome) that restore functional gene activity necessary to survive and replicate in earthworms, rhizosphere, biofilms, and soil[6].

Soil has been hypothesized to help aid dispersion and protection of *B. anthracis* spores in the environment. Spores have been found to move between solid surfaces and water and tend to be in water[7]. Spore transport might also occur during flooding, during natural drainage, and due to reaerosolization of the spores from soil caused by wind.

In an effort to better characterize anthrax distribution throughout the world, researchers have also been working to develop better methods to characterize occurrence. For example, genomic analysis techniques have been used more recently to track *B. anthracis* genetic sublineage in order to understand genetic-ecological associations and to construct ecological niche models to predict natural outbreaks and to rapidly identify intentional anthrax events[8]. However, identifying *B. anthracis* spores in an environmental sample continues to be a difficult task due to the inhibiting compounds within a soil matrix, low concentrations of the spores themselves, and low processing efficiencies. The lack of efficient methods to isolate *B. anthracis* spores from soil has affected the ability of scientists to accurately characterize the persistence of the spores in

[4] Ganz et al. 2014. *PLOS Neglected Tropical Diseases* 8(6): e2903.
[5] Dey *et al*., 2012. *Appl Environ Microbiol* **78** (22): 8075-8081.
[6] Schuch and Fischetti, 2009. *PLoS One* **4** (8): e6532.
[7] Chen et al. 2010. *Colloids Surf B Biointerfaces* **76** (2): 512-518.
[8] Mullins et al. 2011. *BMC ecology* **11** 32; Kracalik et al. 2013, *PLoS Negl Trop Dis* **7** (9): e2388; Mullins et al. 2013, *PLoS One* **8** (8): e72451.

nature. Work on improving the recovery of *B. anthracis* from soil and other matrices is continuing.

Several knowledge gaps, as identified by this review, need to be addressed in order to understand the human risk associated with *B. anthracis* residual contamination and persistence of spores in the environment. The gaps include, but are not limited to, the correlation of non-host microenvironments on persistence, factors affecting sporulation, location of spores during dormancy periods, natural attenuation of *B. anthracis*, and how readily virulence plasmids are lost and regained in nature.

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List of Acronyms and Abbreviations

CFU	Colony forming units
DNA	Deoxyribonucleic acid
EPA	U.S. Environmental Protection Agency
GABRI	Ground anthrax *Bacillus* refined identification
ML	Milliliter
MLVA	Multiple locus variable tandem repeat analysis
SASP	Small acid-soluble proteins
UV	Ultraviolet

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1. Introduction

Anthrax outbreaks have occurred for thousands of years, and have been hypothesized to have caused the fifth and sixth plagues as described in the Bible, in Exodus, Chapters 7-9 (Hugh-Jones and de Vos 2002). Robert Koch first described the etiology of anthrax in 1876, and significant work has been devoted to the organism in the years since (Fennelly et al. 2004; Hudson et al. 2008). Risk associated with the presence of *B. anthracis,* the causative agent for anthrax, in the environment in the United States, depends on a variety of bioclimatic factors such as soil type, environmental conditions, and ecology, as well as bacterial lifecycle, persistence, and potential to lose (or gain) virulence.

A key consideration in understanding the environmental impact of *Bacillus anthracis* cells is the stability of the organism in a variety of matrices. Persistence is the ability of an organism to remain viable (e.g., to remain alive) over time under a given environmental condition and medium. While the vegetative form of *B. anthracis* is not expected to persist in soil as it does not compete well with other microorganisms in nature (Titball et al. 1991; Hugh-Jones and Blackburn 2009), when they are exposed to certain conditions, such as contact with air, they rapidly sporulate to form very persistent spores (Hudson et al. 2008). Once formed, *B. anthracis* spores can resist prolonged periods of desiccation, extremes in temperature, pressure, pH, ionizing radiation, and ultraviolet (UV) radiation, allowing spores to potentially survive for hundreds of years (Dragon and Rennie 1995).

B. anthracis persistence also depends on chemical and environmental characteristics as well as surviving the competition from other organisms (Minett and Dhanda 1941; Van Ness 1971). The There have been numerous examples of extended *B. anthracis* stability in the environment. For example, in pond water, *B. anthracis* spores have been reported to survive 18.5 years (Minett and Dhanda 1941); in moist or dry soil for years (3 months to 33 months) (Manchee et al. 1981; Sinclair et al. 2008; Hugh-Jones and Blackburn 2009); in soil or gravel at anthrax carcass sites for several years to decades (Manchee et al. 1981; Turnbull et al. 1998; Sinclair et al. 2008). *B. anthracis* has also been found to survive for 10 to 22.5 years on canvas held at room temperature, with low humidity and diffuse sunlight and was found to be viable up to 40 years within dried blood on gauze (Army Biological Defense Research Center 1953). *B. anthracis* spores persist best in dry conditions with soils that are relatively alkaline (above pH of 6), high in calcium, and high in organic matter (Dragon and Rennie 1995; Hugh-Jones and Blackburn 2009).

Anthrax is endemic for livestock living in warmer climates, including the United States (Van Ness and Stein 1956; Dragon and Rennie 1995). As such, livestock are seen as a primary source of *B. anthracis* in the environment. Globally, human outbreaks of anthrax due to environmental exposure are widespread (Kracalik et al. 2013). Humans are susceptible to anthrax infection from

three routes of exposure: cutaneous (by introduction of spores into a lesion or by an insect bite transmitting spores from an infected animal); gastrointestinal (by ingesting contaminated meat or water); and pulmonary (by inhaling spores) (WHO 2008). Johnson (2007) has developed a list of criteria to differentiate between natural and non-natural epizootics of anthrax in livestock populations after an event (an outbreak or an attack) has occurred. Blackburn et al. (2007) has developed an ecological niche modeling tool to predict the geographical distribution of *B. anthracis* across the continental United States prior to observed outbreaks by using historic wildlife and livestock outbreak data along with several environmental variables. Their study depicts a significant corridor of increased *B. anthracis* presence running north to south from Canada to Mexico. Griffin et al. (2009) was able to confirm the existence of *B. anthracis* isolates within a similar transect of North American soils. The identified areas follow historical cattle trails (Blackburn et al. 2007). In many instances, recent anthrax cases are associated with old graves of anthrax stricken animals and suitable soil conditions (Pepper and Gentry 2002; Griffin et al. 2009; Hugh-Jones and Blackburn 2009; Kracalik et al. 2013).

Assessing risk associated with the presence of *B. anthracis* in the environment in the United States, whether as background levels, during an outbreak, or as residual levels after an act of bioterrorism, might depend on a variety of factors such as soil type, environmental conditions, lifecycle, persistence, ecology, and potential to lose (or gain) virulence. The present study provides a literature review of the state of knowledge with respect to the persistence of *B. anthracis* vegetative cells and spores in environmental soils. Knowledge gaps are also identified in this review.

2. Purpose

The purpose of this study was to determine the current state of scientific knowledge regarding the persistence of naturally occurring *B. anthracis* spores in soil matrices. A discussion of persistence of *B. anthracis* vegetative cells has also been included for completeness. Within this document, persistence refers to spores or vegetative cells that are viable and culturable. The ability of these cells to subsequently cause infection or induce human disease following release into the environment is beyond the scope of this review.

3. Methods

Information about *B. anthracis* for this literature review was considered from reports, peer-reviewed journal articles, books, and government publications focusing on publications in the last ten years, but including relevant information dating back to the 1940s from open literature. Books were limited to those published or revised in the last 10 years. The primary search engine used was Google Scholar™ ; Pubmed® and Chemical, Biological, Radiological, and Nuclear Defense Information Analysis Center were used secondarily. Search terms included the agent name plus "persistence," "survival," "viability," and "recovery." The review focused on the environmental persistence associated with soil. The search was limited to articles published in the English language, but there was no restriction on geographic location. Additional search terms were used in conjunction with the agent name and "persistence," "survival," "viability," and "recovery" as a check to ensure important variables affecting the agent's persistence were captured including: soil type, geochemical properties of the matrix, rhizosphere, native organisms, soil pH, weather patterns, moisture, temperature, relative humidity, time, UV light and solar radiation.

This report was generated using references (secondary data) that could not be evaluated for accuracy, precision, representativeness, completeness, or comparability and therefore no assurance can be made that the data extracted from these publications meet the U.S. Environmental Protection Agency (EPA) quality assurance requirements. However, the sources of secondary data were limited to peer-reviewed documents.

4. Results

Persistence, sporulation of *B. anthracis* vegetative cells, and spore structure. *B. anthracis* vegetative cell persistence depends on chemical and environmental characteristics and on surviving competition from other organisms (Van Ness 1971). The vegetative form of *B. anthracis* does not compete well with other soil microorganisms in nature (Titball et al. 1991; Hugh-Jones and Blackburn 2009). *B. anthracis* forms metabolically dormant and extremely resistant spores as a survival mechanism when vegetative cells experience nutrient-limiting conditions (Ghosh and Setlow 2009; 2010). These environmentally resistant spores are composed of a series of concentric layers that each has a role in extending the persistence of the organism (Koehler 2009). At the center is the core, where the chromosome along with tightly bound small acid-soluble proteins (SASPs) are found. High levels of calcium dipicolinic acid along with the SASPs protect the core deoxyribonucleic acid (DNA) from UV degradation (Driks 2009). A salt lattice structure formed between the calcium and the dipicolinic acid stabilize the DNA and enzymes within the core increasing the thermoresistance properties of the spore (Himsworth 2008). The membrane and peptidoglycan cortex layers surround the core, and work together to keep the core dry (Driks 2009). Finally, two protein layers called the spore coat and exosporium surround the cortex of *B. anthracis* spores (Driks 2009). The coat prevents foreign materials from entering the core (Driks, 2009). The exosporium is present in several *Bacillus* species, including members of the *B. cereus* group. It is composed of a basal layer and a hair-like nap layer made up of glycoproteins which interact with the environment to signal when conditions are optimum for germination (Driks 2009; Kailas et al. 2011; Thompson et al. 2011).

The structure of the exosporium and its nap coat are thought to aid in binding spores to charged particles in soil, and thereby influence the dispersion of *Bacillus* spp. spores (Hugh-Jones and Blackburn 2009). Multiple works show that both soil characteristics and spore exosporium structures play a role in how well a spore will adhere to its surroundings. Williams et al. (2013) found that while the presence of the exosporium impacts the adherence to surfaces, the impact is less noticeable on steel than in soil. Taylor–McCabe et al. (2012) correlated the recovery efficiency of *B. anthracis* spores from porous (sand) and non-porous (clay) soils to DNA signature recovery. They found that, in general, the more hydrophobic the exterior of a spore strain the better adhesion the spore will have to its surrounding environment. *B. anthracis* spores have been found to have higher cell surface hydrophobicity and the least negative electrophoretic mobility compared to other commonly used surrogates (White, et al. 2012; White et al. 2014). It has been hypothesized that the high hydrophobicity and more negative charge of the spores in higher pH ranges (such as alkaline soil) prevents loss of calcium from the spore core and thus maintain germination capability and viability (White et al. 2012; White et al. 2014). Work looking at leachate from simulated landfills confirms that the hydrophobic nature of the *B. atrophaeus* spores used in the study retarded their transport through the simulated landfills

(Saikaly et al. 2010). Furthermore, Chen et al. (2010) determined that functional groups on the spore surface significantly influence transport properties, and thereby also influence the persistence of *B. anthracis* spores in nature.

Spore physiology influences the resistance and dispersion of *B. anthracis* spores. In particular, the metal ions available during sporulation have been found to influence spore characteristics. *Bacillus* spp. spores enriched with calcium germinated more quickly than spores enriched with strontium or barium (Himsworth 2008).

Initiation and speed of *B. anthracis* sporulation is also dependent on temperature and relative humidity (Minett 1950; Hugh-Jones and Blackburn 2009). Minett (1950) reported that sporulation associated with open carcasses is dependent upon temperature, with sporulation rates increasing as temperatures increase; at 36°C sporulation occurs in 8 hours, at 32°C by 10 hours, at 26°C by 18 hours, and at 21°C by 24 hours. The EPA (2014) found that the vegetative cell population grew by a factor of 10 and had extensive sporulation on wet topsoil within 48 hours when held at room temperature and high relative humidity. When an inoculum contained *B. anthracis* in both vegetative cell and spore form, both cells and spores were recovered from the test materials 56 days after inoculation. While a temperature of 39°C and high relative humidity (100%) has been cited as being ideal for sporulation to occur (Hugh-Jones and Blackburn 2009); sporulation in soil is reported to only occur at temperatures above 25°C (Lindeque and Turnbull 1994). At a temperature of about 20°C, sporulation is inhibited and the vegetative form of *B. anthracis* dies (Hugh-Jones and Blackburn 2009).

While the spore possesses numerous protective properties, some studies show that it is still susceptible to environmental degradation. Laboratory studies conducted by Minett (1941) have shown that exposure to sunlight can kill a spore within 84 hours. UV radiation at 452 erg/m^2 (45.2mJ/m^2) and 2537Å (253.7 nm) destroys 90% of cells (Army Biological Defense Research Center 1953). However, environmental soils might have a UV A/B protective effect for *B. anthracis* cells. EPA (2010) compared the persistence of *B. anthracis* spores spiked onto topsoil, glass, bare pine wood, and unpainted concrete that had been exposed to simulated sunlight (UV-A/B). Results determined that when exposed to simulated sunlight (UV-A/B) there was essentially no loss in recovery of *B. anthracis* spores on topsoil after 28 days, while there was over a 5 log reduction of spores on glass for the same time period (EPA 2010).

In a more recent study, work with topsoil determined that *B. anthracis* cells did not sporulate and were culturable for up to 96-120 hours at normal laboratory temperatures and a relative humidity of 46% regardless of UV-A/B (simulated sunlight) exposure (EPA 2014).

***B. anthracis* genetics and persistence**. *B. anthracis* is thought to have been derived from the *B. cereus* and *B. thuringiensis* clade (Okinaka 2006) and is considered to be closely related to members of the *B. cereus* group (Klee et al. 2010; Greenberg et al. 2010). *B. cereus* is a saprotrophic, opportunistic pathogen commonly found in the rhizosphere and as a gut commensal (Schuch and Fischetti 2009), while *B. thuringiensis* is a common insect pathogen that has been utilized for decades as a natural pesticide (Tilquin et al. 2008; Van Cuyk 2011). Each of these organisms share a core set of genes that support growth within a diverse range of environments. The transcriptional regulator *plcR* regulates over 100 loci, which allows organisms such as *B. cereus* and *B. thuringiensis* to respond to the environment (Schuch and Fischetti 2009). However, although the loci are encoded for in *B. anthracis*, a single nonsense mutation has caused an inactivation of *plcR* and transcriptionally silenced these loci in *B. anthracis*, thus allowing the capacity for survival in the environment (Schuch and Fischetti 2009). It is thought that all members of the *B. cereus* group share a pan genome within which select portions, specifically prophages, are exchanged through horizontal gene transfer (Klee et al. 2010). Thus, *B. anthracis* carries the genetic capacity for environmental survival and transferred prophages could modify the expressed phenotype to promote environmental persistence (Schuch and Fischetti 2009).

In an effort to stay at the forefront of anthrax distribution throughout the world, researchers have begun using genomic analysis techniques to track *B. anthracis* genetic sublineage (Aikembayev et al 2010; Kenefic et al. 2008; Mullins et al. 2013). The hope is that by understanding genetic-ecological associations, adequate ecological niche models can be constructed to predict natural outbreaks and rapidly identify anthrax events begun from illicit mechanisms (Mullins et al. 2011; Kracalik et al. 2013; Mullins et al. 2013). While *B. anthracis* has a limited global diversity, multiple locus variable number tandem repeat analysis (MLVA) systems can differentiate *B. anthracis* into lineages and sublineages (Mullins et al. 2011) and single nucleotide repeat markers can be used to give a detailed analysis in an outbreak using a fine scale resolution (Kenefic et al. 2008). Global studies of *B. anthracis* have shown that the A lineage is globally distributed, while the B and C lineages are more limited in geographic distribution (Van Ert et al. 2007). The adaptive differences between the three lineages might have aided in the dispersion and persistence of *B. anthracis* worldwide.

A comparison of A and B lineage isolates collected from Kruger National Park, South Africa found that the B strains were only found in soils with significantly higher calcium concentrations and soil pH measurements when compared to the A strains (Smith et al. 2000; Hugh-Jones and de Vos 2002). In fact, the large genetic differences found among samples collected from Kruger National Park have led some to hypothesize that southern Africa might be the geographic origin of *B. anthracis* (Smith et al. 2000). Genotyping work in the Central Asian nation of Kazakhstan shows that *B. anthracis* A1.a sublineage was associated with a more diverse ecologic distribution than the other A lineage isolates studied (Mullins et al. 2011). Furthermore, the A1.a sublineage

is the prevalent *B. anthracis* lineage across the United States, Italy, and Kazakhstan (Van Ert et al. 2007; Aikembayev et al. 2010; Fasanella et al. 2010; Mullins et al. 2011). Results of ecological niche modeling studies characterizing the A1.a sublineage across theses three countries suggest that specialization within the A1.a sublineage might have occurred over time (Mullins et al. 2013). This genetic specialization for various geographic locations has pointed to the need for regional ecological niche models that account for genetic diversity of the *B. anthracis* lineage(s) present in conjunction with soil and climate characteristics (Mullins et al. 2013). Such knowledge has recently led researchers to determine that an anthrax outbreak in Bangladesh was most probably due to the domestic recycling of bone as bonemeal for livestock feed rather than contaminated soils. Fasanella et al. (2013) recovered three distinct MLVA genotypes of the sublineage A.Br.001/002 from six geographically close herds that primarily stay on clay soils. Work by Ahsan et al. (2013) furthered the findings by Fasanella et al. (2013) by collecting additional soil samples from low lying areas, livestock pastures, and burial sites near the original sampling sites. Fourteen of the 48 samples collected by Ahsan et al. (2013) were positive for *B. anthracis* spores. None of the clay samples were positive for *B. anthracis*, but rather all of the spore positive samples were from loamy soils with an elevated moisture content (16.69±2.06%) and slightly acidic pH (6.38±0.15) (Ahsan et al. 2013). Kenefic et al (2008) found multiple SNR genotypes and a random spatial and temporal pattern of isolates of *B. anthracis* after taking blood and tissue samples from 47 cattle that were associated with an outbreak in South Dakota in 2005. His results suggested that the area was associated with multiple past outbreaks and conditions that favored spore survival. Together these reports indicate that while anthrax could be occurring at a higher rate due to contaminated feedstock, the area also has a natural propensity to harbor *B. anthracis* spores in select areas.

***B. anthracis* lifecycle and environmental persistence.** Members of the *Bacillus* genus are primarily saprophytes (organisms that obtain nutrients from dead matter) living in soil (Saile and Koehler 2006). In contrast, *B. anthracis* has traditionally been considered an obligate pathogen, unable to reproduce outside of an infected host in nature due to a single nonsense mutation that inactivated a transcription regulator gene attributed to saprophytic capabilities (WHO 2008; Schuch and Fischetti 2009). Establishment of a persistent cycle from a host, to soil, to a host has been seen as the basis for recurrent outbreaks (Van Ness 1971). The classic *B. anthracis* lifecycle (Figure 1) involves:

- a period of prolific growth (on the order of multiple millions of vegetative organisms per milliliter of blood within an infected individual, producing toxins that kill the host)
- rapid (condition dependent) sporulation at the carcass site initiated when predation (or other events) open a carcass, allowing the bodily fluids to drain from the infected carcass
- dispersion of vegetative cells into the environment (air, soil, or water)
- spore acquisition by a new host
- spore germination in a new host (Lindeque and Turnbull 1994; Schuch and Fischetti 2009)

B. anthracis is present at high levels, a million to a billion CFU/mL in the blood of animals when they die of anthrax (Lindeque and Turnbull 1994). High carbon dioxide concentrations that are found in decomposing carcasses reduce sporulation. *B. anthracis* in carcasses die in four days or less under conditions supporting anaerobic digestion (Minett 1950; Hugh-Jones and Blackburn 2009) and under conditions where they are in competition with other microorganisms (Dragon and Rennie 1995). For sporulation to occur, carcasses must be opened, or bodily fluids drained from the carcass (Minett 1950; Johnson 2007; Hugh-Jones and Blackburn 2009). Predators are responsible for opening carcasses, and thereby facilitating sporulation (Dragon et al. 2005). In locations and/or conditions not conducive to sporulation, spilled blood of infected animals with high *B. anthracis* colony forming units (CFU) per milliliter (mL) could result in few or no spores in soil as vegetative *B. anthracis* survival outside of a host is poor (<24 h) (Lindeque and Turnbull 1994; Atlas 2002).

Anthrax outbreaks have been known to stop occurring for decades at a site before an herbivorous (typically) host again becomes infected and the cycle is repeated (Hugh-Jones and Blackburn 2009). Details of natural *B. anthracis* cycles during the long dormancy periods and explanations for the initiation of outbreaks after dormant periods are incomplete and controversial. For an area to provide a risk of anthrax, virulent *B. anthracis* must be present. *B. anthracis* is most often found in dry conditions with soils that are high in organics and calcium, and are relatively alkaline (above pH 6) (Van Ness 1971; Hugh-Jones and Blackburn 2009; Aikembayev et al. 2010). In a study that aimed to map the spatial and temporal distribution of anthrax outbreaks in Kazakhstan, foci for anthrax were concentrated more in regions with alkaline soil and higher organic matter (southern and areas in northern regions) compared to areas that consisted of desert and poorer soil conditions (central Kazakhstan) (Aikembayev et al. 2010). In a review by Sinclair et al. (2008) it was noted that the lifecycle of *B. anthracis* includes a soil dwelling stage, and that viable spores can be recovered after significant periods (40, 60, and potentially 1,000 years). However, while the potential for germination and multiplication of *B. anthracis* in the soil dwelling stage were mentioned, the mechanism and conditions favoring this germination were not discussed in the Sinclair et al. (2008) review.

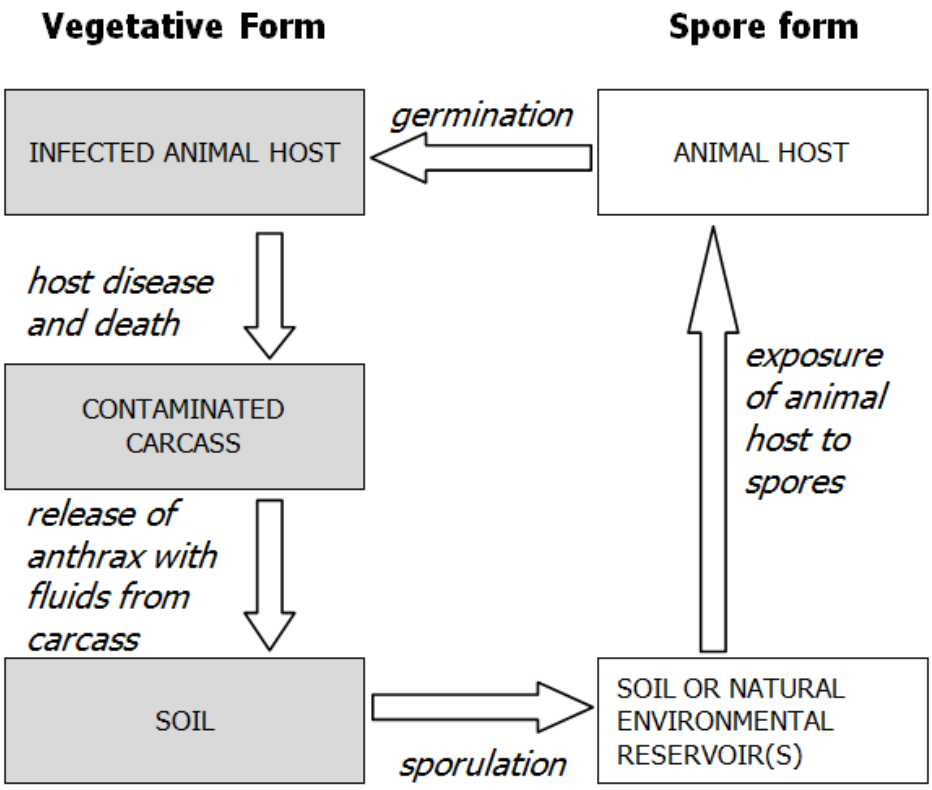

Figure 1. *B. anthracis* natural lifecycle; modified from Schuch and Fischetti (2009).

The concept that spores germinate and propagate in soil is not new. West and Burges (1985) spiked spores of *B. thuringiensis* and *B. cereus* into sandy silt loam soils supplemented with either grass clippings or chicken manure. Their results showed that *B. thuringiensis* spores germinated and propagated in the grass-supplemented soil, but *B. cereus* did not. After 24 days, the *B. thuringiensis* counts came to a plateau and remained at 22 times the spore inoculum level for the duration of the experiment, while *B. cereus* had an initial germination spike then declined to 0.11 times the inoculum. Similarly, *B. thuringiensis* and *B. cereus* spores germinated in manure-supplemented soil; however, after the initial burst of activity the viability counts significantly decreased (West and Burges 1985). These results indicate that though spores might be activated by an abundance of fresh nutrients in supplemented soils, germinated cells do not readily persist in all soils (West and Burges 1985). Other works have also suggested that *B. thuringiensis* can proliferate in nature under ideal conditions. Tilquin et al. (2008) found that *B. thuringiensis* subspecies *israelensis* spores, originally sprayed in France as a means of mosquito control, were found in high concentrations in leaf matter (3×10^5 spores g^{-1}). The authors suggest that the high concentrations of *B. thuringiensis* found might be because the leaf litter is a specific microenvironment in which the organism can persist and grow. Specifically, the work highlighted two key elements to the microenvironment that potentially contributed to the increase in spore counts– low oxygen levels and low decomposition rates (Tilquin et al. 2008). While these studies did not utilize *B. anthracis,* their work is important because both *B. thuringiensis* and *B. cereus* are routinely used as surrogates for *B. anthracis*.

***B. anthracis* dispersion and persistence in nature**. It has been hypothesized that soil might aid in both dispersing and protecting *B. anthracis* spores in nature (Williams et al. 2013). Within this theory, soil surrounding the spores limits UV A/B radiation exposure (EPA 2010), while spore-soil binding influences spore dispersion patterns. Work by Chen et al. (2010) determined that *B. anthracis* Sterne spores are monopolar and negatively charged. These findings indicate that in natural environments where spores can move between a solid surface and water (i.e. in soil near a body of water), spores will tend to be in water much of the time (Chen et al. 2010). This conclusion corroborates findings by Kim et al. (2009) who determined that an increase in water flow rate has the greatest effect on spore movement. Not only will spores tend to be in water, but also as the water velocity increases, hydrodynamic forces acting on the soil surface increase, which results in the decrease of interparticle bonding forces and increased spore movement. Thus, periods of local flooding which raises the water table could detach spores from their refuge and transport them back to the soil surface, contaminating the vegetation grazed by susceptible herbivores, and thus begin the *B. anthracis* lifecycle again (Kim et al. 2009; Williams et al. 2013).

Spore transport during local flooding and natural drainage could also account for limited horizontal dispersion patterns (Manchee et al. 1994; Hendriksen and Hansen 2002; Hugh-Jones

and Blackburn 2009; Kim et al. 2009; Williams et al. 2013) and areas of higher spore concentrations in the environment (Van Ness 1971; Dragon et al. 2005; Griffin et al. 2009; Williams et al. 2013). Van Ness (1971) hypothesized that spores germinate and proliferate following a flood event then quickly re-sporulate. Dragon and Rennie (1995) called areas of soil with high spore concentrations "storage areas." In their assessment, rather than reproducing, hydrophobic spores were transported to low-lying areas where they were concentrated as the soil dried out and left to interact with plant cuticles, increasing exposure of grazing animals to the spores (Dragon and Rennie 1995). While a connection between local flooding and anthrax incidence has been recognized, the exact role in dispersion and persistence of *B. anthracis* spores is still uncertain.

The role wind and ground perturbation plays in dispersing *B. anthracis* spores from soils has been assessed (Layshock et al. 2012). In 1949, military researchers sprayed grassy fields with either a dry powder or wet slurry of *B. anthracis* before collecting air samples while troops marched through the fields. Their results showed minimal reaerosolization from the troop activity (Peck 1949). Aerosol samples have also been collected downwind from anthrax carcass sites (Turnbull et al. 1998). It was found that *B. anthracis* spores were only found downwind of the carcass site during periods of manual disturbances and with the highest wind gusts. These results led Turnbull et al. (1998) to conclude that spores are mostly attached to large soil particles, and do not remain airborne for very long. Work conducted by Byers et al. (2013) in a laboratory controlled ambient breeze tunnel determined that the reaerosolization rates of spores are dependent upon the humidity, temperature, and particle size.

***B. anthracis* and non-host microenvironment effect on environmental persistence**.
Several studies have focused on the ability of *B. anthracis* to propagate in soil environments. In one such work, *B. anthracis* was found to propagate within the rhizosphere of a common pasture grass (Saile and Koehler 2006). The authors proposed that the plant roots enhanced germination and proliferation of vegetative cells, as nearly 50% of the inoculated spores germinated in the presence of plant roots, whereas few to no spores germinated in the absence of the grass (Saile and Koehler 2006). Work conducted by Kiel et al. (2009) has demonstrated the ability of *B. thuringiensis* to grow within rhizome nodules of fescue grass, and a hybridizing relationship between a barley flavoprotein gene and a nitrate reductase gene of *B. anthracis*. Studies have also found evidence of plasmid transfer between two strains of *B. anthracis* within the model rhizosphere system (Saile and Koehler 2006). This finding is significant as it provides evidence of metabolically active *B. anthracis* cells in the plant-soil environment. Together these findings have led Kiel et al. (2009) to hypothesize that growth of *B. anthracis* within rhizome nodules could be the missing environmental reservoir of *B. anthracis* between natural anthrax outbreaks. However, conflicting results were found by Ganz et al. (2014) who aimed to test whether interaction of *B. anthracis*, native grass (*Enneapogon desvauxii*), and carcass blood increased *B. anthracis* survival in soils from Etosha National Park. The results suggested that while *B.*

anthracis might increase the transmission of *B. anthracis* to grazing hosts by promoting establishment of grass at carcass sites, which attracts the hosts, no evidence was found that the presence of the grass increased *B. anthracis* survival or multiplication in the soil (Ganz, 2014).

There have been a number of studies looking at potential commensal or symbiotic relationships between *Bacillus* species and soil borne organisms, including amoeba, nematodes, earthworms, and bacteriophages. Dey et al. (2012) simulated a stagnant water/moist soil environment to assess the interaction of *B. anthracis* (Ames and Sterne) with *Acanthamoeba castellanii*, a common soil amoeba. The work showed a pathway where ingested *B. anthracis* spores germinate within the amoeba and replicate to the point of lysing the amoeba host. Upon lysis, the vegetative cells sporulated in the simulated stagnant water/moist soil environment. Their work also highlighted the importance of the pX01 plasmid. Virulent *B. anthracis* contains two plasmids, pX01 and pX02. Interestingly, a pX01 plasmid was required for spore germination within the amoeba (Dey et al. 2012). Similar findings were noted in soil samples collected in New Orleans after Hurricane Katrina. Of the five *B. anthracis* positive samples collected post-flood, all had the pX01 plasmid and only one contained both pX01 and pX02 plasmids (Griffin et al. 2009). Researchers hypothesized that this germination dependence could explain why pX02 is lacking in multiple natural *B. anthracis* strains – it is not required for proliferation (Dey et al. 2012). There is some evidence that virulence plasmids can be acquired or reacquired to induce virulence. For instance, *B. cereus* induced an anthrax-like disease in multiple chimpanzees in the Ivory Coast. Both pX01 and pX02 virulence plasmids were found in the *B. cereus* genome in these animals (Klee et al. 2010). Researchers hypothesized that the virulence plasmids were obtained from *B. anthracis* cells in the soil or another animal that was co-infected with *B. anthracis* and *B. cereus* (Klee et al. 2010).

The intestinal tract of worms has been explored as a potential site of *B. anthracis spore* germination. Laaberki and Dworkin (2008) fed *B. anthracis* Sterne spores and vegetative cells to laboratory-controlled nematodes. They found that the digestive track of the worms killed the consumed vegetative cells, but spores were able to pass through their system into their feces and remain viable. This conclusion is supported by work conducted by Hendriksen and Hansen (2002). However, Hendriksen and Hansen (2002) were able to determine that consumed *B. thuringiensis* spores germinated in the gut of the earthworms prior to re-sporulation and defecation. Furthermore, their study showed that *B. thuringiensis* was able to germinate in the hindgut of three of the four tested earthworm species, indicating that this is not a species limited occurrence, but rather a widely distributed capability (Hendriksen and Hansen 2002).

More recently, the interaction between earthworms and *B. anthracis* has been shown to be dependent upon the presence of various bacteriophages (viruses that infect bacteria). Bacteriophage infection of *B. anthracis* could create lysogens (the integration of phage nucleic acid into the bacterial chromosome) that restore the functional gene activity necessary for the

bacteria to survive and replicate in earthworms, rhizosphere, biofilms, and soil (Schuch and Fischetti 2009). These lysogens appear to induce phenotypic changes to the *B. anthracis* vegetative cells that alter their capacity to sporulate, produce exopolysaccharide for biofilm production, and survive long-term in soil and in the anoxic earthworm gut (Schuch and Fischetti 2009; Schuch et al. 2010). Research has shown that only bacteriophage-infected *B. anthracis* are capable of infecting the intestinal tract of earthworms, and that the bacteriophage present are variable (Schuch and Fischetti 2009; Schuch et al. 2010). Work by Schuch et al. (2010) showed that one bacteriophage, worm intestinal phage 1, was present in high numbers for three years in Pennsylvania forest soil before the population was replaced by a second bacteriophage, worm intestinal phage 4, for the next three years of the study. This demonstrates that the bacteriophage population in environmental *B. anthracis*-like isolates in earthworm intestinal tracts is variable.

Another point of interest is the comparison of the earthworm lifestyle to what is known of the *B. anthracis* lifecycle in soils. Both earthworms and *B. anthracis* prefer alkaline soils with high calcium concentrations and rich in organics (Hugh-Jones and Blackburn 2009; Schuch et al. 2010). This lifestyle correlation might be an indication as to why significant spore spreading is not seen in some locations. For instance, after 40 years the spore spread in the acidic soils of Gruinard Island was limited to the top 6 cm of soil surrounding the original detonation point (Manchee et al. 1981; Manchee et al. 1994; Sharp and Roberts 2006). Furthermore, even the limited horizontal distribution seen after 40 years was attributed to wind and/or drainage from the contaminated areas, not soil borne organisms (Manchee et al. 1994; Hugh-Jones and Blackburn 2009). Anthrax events also seem to occur more commonly after seasonal flooding events when earthworms seek the soil surface (Griffin et al. 2009; Schuch et al. 2010). The combination of lifestyle patterns, hindgut colonization, and anthrax occurrence patterns points to a significant relationship between anthrax incidence and earthworms. While direct sampling at enzootic areas would be required to definitively determine a correlation between earthworms and anthrax outbreaks, their overlapping lifestyles suggests that *B. anthracis* spores are carried upward to the soil surface and potentially onto vegetation via colonized earthworm digestive systems.

***B. anthracis* biofilms and persistence.** Under appropriate conditions, *B. anthracis* readily forms biofilms (Lee et al. 2007; Auger et al. 2009; Schuch and Fischetti 2009). Biofilms are complex communities of microbes that produce glycocalyx polysaccharides to protect them from desiccation, chemical treatment, immunological attack, and antibiotics (Lee et al. 2007; Auger et al. 2009; Perkins et al. 2010). Biofilms are well known to harbor infectious organisms, lengthen persistence, and aid in dispersion through sloughing of fringe layers (Perkins et al. 2009). Due to their structure, biofilm microenvironments have been hypothesized to create a more suitable environment for vegetative *B. anthracis* to flourish and engage in genetic transfer in nature (Lee et al. 2007).

As mentioned previously, the ability of *B. anthracis* to form biofilms could have a profound impact on environmental persistence and proliferation. Biofilm formation can create areas of nutrient limitation, thereby triggering spore formation for the *B. anthracis* cells (Lee et al. 2007). It has also been found that when biofilms are supplemented with carbon dioxide during culture, the vegetative cell concentration is higher compared to ambient temperature and pressure conditions (Lee et al. 2007). Studies have shown that *B. anthracis* strains involved in gut colonization are adept at forming biofilms (Auger et al. 2009). This finding again points to the symbiotic relationship between *Bacillus* species and soil borne organisms.

***B. anthracis* sampling methods and persistence.** Identifying *B. anthracis* spores in an environmental sample can be a difficult task due to inhibiting compounds within a soil matrix, low concentrations of the spores themselves, and low processing efficiencies. Such sampling difficulties might be one factor for seemingly conflicting results from field sampling efforts. Multiple detection methods have been developed for clean samples, such as, culture analysis, biochemical analysis, genetic analysis, and immunologic analysis (Rao et al. 2010; Irenge and Gala 2012). However, without appropriate sample processing, the most sensitive detection method will be ineffective. Environmental detection limits for *B. anthracis* spores in soil have been estimated to range between 0.1 CFU/g to $3.2x10^8$ CFU/g soil depending upon the approach used to evaluate the environmental limit of detection although limited information is available on which is the best approach (Herzog et al. 2009; Chikerema et al. 2012). As pointed out in a review by Lim et al. (Lim et al. 2005) there is a need for a universal sample processing method to separate, concentrate, and purify target agents from any sample type. The lack of efficient methods to isolate *B. anthracis* spores from soil has affected the ability of scientists to accurately characterize the persistence of the spores in nature. A fully developed method with a known method recovery rate and associated confidence intervals would be useful for determining the persistence of *B. anthracis* spores, their viability, and the extent of their presence in the environment.

5. Discussion

As summarized in this report there are numerous concepts regarding how and where *B. anthracis* spores persist in the environment. The evidence included here points to multiple specific microenvironments that allow *B. anthracis* with suitable genetic phenotypes to germinate and re-sporulate in the environment, and thereby both increase in concentration and allow for genetic transfer (Saile and Koehler 2006; Lee et al. 2007). These microenvironments include amoebae, biofilms, earthworm intestinal tracts, and grass rhizospheres (Saile and Koehler 2006; Lee et al. 2007; Schuch et al. 2010; Dey et al. 2012). Spores generated through these microenvironments might be sufficient to carry the species until suitable conditions are met for prolific germination, such as in nutrient supplemented soils or a mammalian host (West and Burges 1985; Tilquin et al. 2008; Schuch and Fischetti 2009). Cell concentrations might then quickly dissipate after

environmental conditions are no longer favorable (West and Burges, 1985). Between anthrax incidents, spores generated through various microenvironments might be concentrated in the soil by local flooding events (Kim et al. 2009; Williams et al. 2013). The ability of *B. anthracis* to propagate in these microenvironments suggests that it is not an obligate mammalian parasite, however, more information is needed to make this determination.

While much of the research of these alternative vegetative pathways for *B. anthracis* has been conducted in laboratory settings, the fact that earthworms, grasses, and *B. anthracis* spores all prefer organic-rich alkaline soils with significant calcium concentrations supports the results of laboratory findings (Schuch and Fischetti 2009; Joyner et al. 2010). A lack of confirmatory field sampling might be attributed to inefficient sampling methods for environmental aliquots with low *B. anthracis* concentrations and high background organism concentrations (Lim et al. 2005). More work focusing on environmental soils with its associated invertebrates and rhizosphere are needed to elucidate the role these identified alternative vegetative pathways have in harboring and distributing *B. anthracis* spores between outbreaks.

The studies highlighted herein point to a combination of genetic, ecologic, and meteorologic factors that allow *B. anthracis* spores to survive in the environment. In one recent study, Kracalik et al. (2013) concluded that ecological factors and anthropogenic factors (lifestyles and farming practices) have a significant role in the persistence of *B. anthracis.* There has also been speculation that human efforts to make land more fertile might contribute to anthrax persistence (Van Ness and Stein 1956). To this end, the Canadian Food Inspection Agency has rescinded its recommendation of using lime (calcium oxide) as an agricultural anthrax disinfectant as lime could aid the long-term preservation of *B. anthracis* spores by providing ample calcium concentrations (Himsworth 2008).

Knowledge gaps in the available literature make it difficult to extrapolate how and where *B. anthracis* persist in environmental soils. These knowledge gaps need to be addressed in order to understand the human risk associated with *B. anthracis* residual contamination and the persistence of *B. anthracis* spores in the environment. Knowledge gaps identified in this literature review include:

- **Sporulation:** What environmental factors are required for sporulation? What is the rate of sporulation under various bioclimatic conditions?
- **Dormancy:** Where are *B. anthracis* spores during anthrax dormancy periods and what interactions occur to break its dormancy? How does anthrax dormancy affect spore persistence?
- **Exosporium:** What role does the spore exosporium structure play in the long-term persistence of a spore?

- **Natural attenuation:** What causes or prevents natural attenuation of *B. anthracis* in the environment?
- **Ecology:** What is the interaction between bacteriophages, worms, and rhizosphere that can induce long-term persistence of *B. anthracis* in soil? How many soil organisms harbor *B. anthracis* spores in their gastrointestinal system? What is the distribution of organisms in various soil types across the United States?
- **Lysogens:** What role do bacteriophage induced lysogens have in the natural *B. anthracis* reproductive cycle?
- **Virulence retention:** How readily are virulence plasmids lost and regained in nature?
- **Biofilms:** What role do environmental biofilms play in the long-term persistence of *B. anthracis*?
- **Detection methods:** What is the best method for detecting *B. anthracis* in soil? What is its limit of detection for multiple soil types?

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