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The Ability of Novel Phage to Infect Virulent *Bacillus anthracis* Isolates

Hyrum Smith Shumway III

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

The Ability of Novel Phage to Infect Virulent
Bacillus anthracis Isolates

Hyrum Smith Shumway III
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Master of Science

Bacillus anthracis is a soil dwelling microbe with pronounced pathogenic potential. Historically, anthrax has infected livestock and man. In the modern-age, anthrax is a bioterrorism concern with major incidents every decade. While the threat of large scale attacks is currently viewed as unlikely, the threat is consistent and constant. Current methods to defend against such an attack focus on antibiotics and containment of public panic. Antibiotic resistance, while not currently an issue for anthrax, could easily become so with genetically engineered weaponized strains created by rogue states or independent actors.

This project evolved from collaborations between the Grose lab and the Robison lab, both housed in the Microbiology and Molecular Biology Department at Brigham Young University in Provo, Utah. Two undergraduates in the Grose lab isolated 23 genetically distinct phage that infect the non-pathogenic *Bacillus anthracis* Sterne strain. Results from spot testing on a diverse library of 11 fully virulent strains that represent the extant genetic diversity of pathogenic *B. anthracis* in BYU's BSL-3 facility give credence to the idea that phage could be useful in containing this pathogen.

Phage were isolated from environmental samples using enrichment culture, high titer lysates of isolated phage were created, and differential assays were performed. Experiments to show phage differences included electron microscopy, restriction digests, and spot testing using different isolates of *B. anthracis*. These data identified several novel phage that could infect a wide variety of virulent *B. anthracis* isolates. Preliminary results also showed most of these phage to be different both morphologically and genetically.We propose that phage therapy deserves further research, public awareness, and increased understanding for governmental regulatory awareness.

Keywords: Phage, pathogenic, anthrax, *Bacillus anthracis*, infection, treatment, bioterrorism, livestock, weaponized, spot testing, antibiotic resistance, plaque, microbe

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Acknowledgments are often done at the end of scientific presentations, but I believe that they should be in the forefront of our thoughts and actions. It is fitting today that the following acknowledgements come early in this thesis as so many individuals played roles both large and small in my project's successes. Without support, no scientist is able to achieve their personal or professional goals. President Obama stated in 2012, "If you were successful, somebody along the line gave you some help. There was a great teacher somewhere in your life. Somebody helped to create this unbelievable American system that we have that allowed you to thrive. . .The point is, that when we succeed, we succeed because of our individual initiative, but also because we do things together." I support this statement as it feels true to my experiences and many family, friends, BYU professors and staff have helped me tremendously.

I am grateful for the support of my family both extended and immediate, friends, and BYU for providing me with the opportunity to work as a student scientist since 2012. Many thanks to the professors in the Microbiology and Molecular Biology Department, outside my committee, at BYU especially Dr. Johnson and Dr. Hope, both for whom I have worked.

Special and specific thanks for my committee of Dr. Robison, Dr. Grose, and Dr. Weber. Dr. Robison who has helped me discover work in biosafety level three and also has been my committee chair and talked with me about a plethora of professional and personal endeavors. Dr. Grose is the professor who helped me determine to study Microbiology and she has been with me since my first class in the department and through my completion of the *B. anthrax* phage project. Dr. Weber has been a caring mentor and whose interactions with I always walk away from uplifted. Dr. Steele in the Department of Nutrition, Dietetics, and Food Science has helped immensely despite his international and domestic travel as I was completing my degree. Thanks

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Thanks to students who worked with me on multiple projects during the course of my Masters training including; Nick Branham, Megumi Wiley, Chris Pace, Hannah Lyons, Alison Curtis, Dan Walton, Taalin Rasmussen, and especially Bram Holladay who has been my longest standing student research assistant. Specific and grateful thanks to students Emily Hansen and Rebecca Eardley for their careful, methodical, and meticulous craftsmanship in finding phage for me upon which to build my project. Without Rebecca and Emily, and working under the careful tutelage of Dr. Julianne Grose, this project would not have been possible. Many thanks as well to other members of the Robison Lab including Alyssa Applegate, Joseph Thiriot, Galen Card, Olivia Brown, Israel Guerrero, Shreena Mody, Anna Reid, and Justen Despain. Also special thanks to my friends Jeralyn Franson, Derek Wade Johanson, Christoph Guynn, and Freddie Hansen for countless calls and support in my personal life.

Most of scientific endeavor could be summed up in the mission of the fictional Star Trek starship, the Enterprise which is: “to explore strange new worlds, to seek out new life and new civilizations, to boldly go where no one has gone before.” Microbiology is a strange new world with its own civilizations, warfare, rivalries, and alliances. Through the course of this project, the team of people who have worked on this project have found new life and have boldly gone where no one has gone before. The project we have built is novel and is important work in the field of bioterrorism, phage, and infectious disease. I am proud to have had the opportunity to be a part of such a great work and to get to know even better people to finish this endeavor.

In my course of “exploring strange new worlds” in Microbiology, I have rediscovered that some of the greatest joys and scientific marvels are the ability for humans to form, create,

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and keep attachments whether at work or personally. Thanks again to any other relationship that was not specifically mentioned who was helpful to me in the process of achieving this project's success. I could not have done this on my own.

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Thanks again to all who make the world a better place. To future successes both in science and without to all investigators of truth. May the world prosper in scientific knowledge, in spiritual and emotional fulfillment, in material wealth, and may nature continue to provide beauty, wonder, and enchantment that delights, astounds, and sparks never ending curiosity to scientists and non-scientists alike.

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BACKGROUND

Bacteriophage

Phage Background

Bacteriophage, or phage, are viruses than infect bacteria (Abedon, 2009). Phage are estimated to be over 3.5 billion years old and represent one of the most common and most biologically diverse entities on earth (H.-W. Ackermann, 1998). Phage are incredible biological tools which have been used as therapeutics to treat bacterial infections and as instrumental research tools. Phage helped elucidate DNA as the genetic material in the landmark experiments of Martha Chase (Chase, 1952; McCallin et al., 2013). Throughout millennia, ancient religious texts of Jews, Hindus, and Christians all encouraged specific ritual washings. Phage may have been important in ancient therapeutic medicine, with people bathing in special bodies of water known for healing, such as the Ganges River in India which has been shown to host therapeutic phage (Khairnar, 2016). Fredrick Twort described and isolated a filterable agent capable of lysing bacteria, and later Felix D'Herelle extensively studied the bacteriophage and showed the therapeutic usefulness of phage in treating illness, especially for patients plagued by dysentery (Doore, Schrad, Dean, Dover, & Parent, 2018; Dublanchet & Bourne, 2007; Gillis & Mahillon, 2014). Phage truly hold great promise in future research as model organisms for molecular biologists, as therapeutics to treat disease, and as a source of novel discoveries related to viral life.

Host Interaction

The two major life states for phage are either lytic or lysogenic. Other intermediate states exist besides lytic and lysogenic, but historically these have been the most significant states associated with the lifecycle of phage (Schuch & Fischetti, 2009). The lysogenic cycle describes

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phage ability to insert their genome into a bacterial host chromosome and become a prophage. The lytic cycle describes the capacity to immediately cause a productive infection in which they use the cell's resources to replicate themselves, lyse the cell, and find new hosts. (Condition & Bacteriophages, 1953). A lytic cycle will destroy large numbers of bacteria while producing even larger numbers of viral progeny.

The lytic cycle occurs in two steps with early genes being expressed that push the cell to upregulate phage replication. When the cell is full of assembled phage, the next step is to express late genes which cause the lytic reaction of viral burst through lysins, holins, and murein inhibitors (Wittebole, De Roock, & Opal, 2013). Much of phage research has been done in gram negative systems. Much less work in the lifecycle and regulation of lytic versus lysogenic phage has been done in gram positive bacteria (Obregón, García, López, & García, 2003).

Figure 1 depicts the lytic vs lysogenic cycle. Phage can also cause horizontal gene transfer (HGT), which is the transfer of genetic material between bacteria. This often complicates genetic tree analyses (Wittebole et al., 2013). HGT can be mediated by conjugation (via sex pili), transduction (through phage) and transformation (the direct uptake of exogenous DNA). Transduction can be either general (any part of a bacterial genome) or specialized (specific parts of a chromosome only) (Gillis & Mahillon, 2014). Multiple transducing events over time can dramatically change the genome of some bacteria. However, this process is much less important in *B. anthracis* as this organism spends most of its time as a spore, which is metabolically inert and incapable of viral replication. (Paul Keim et al., 2004; Schuch & Fischetti, 2009).

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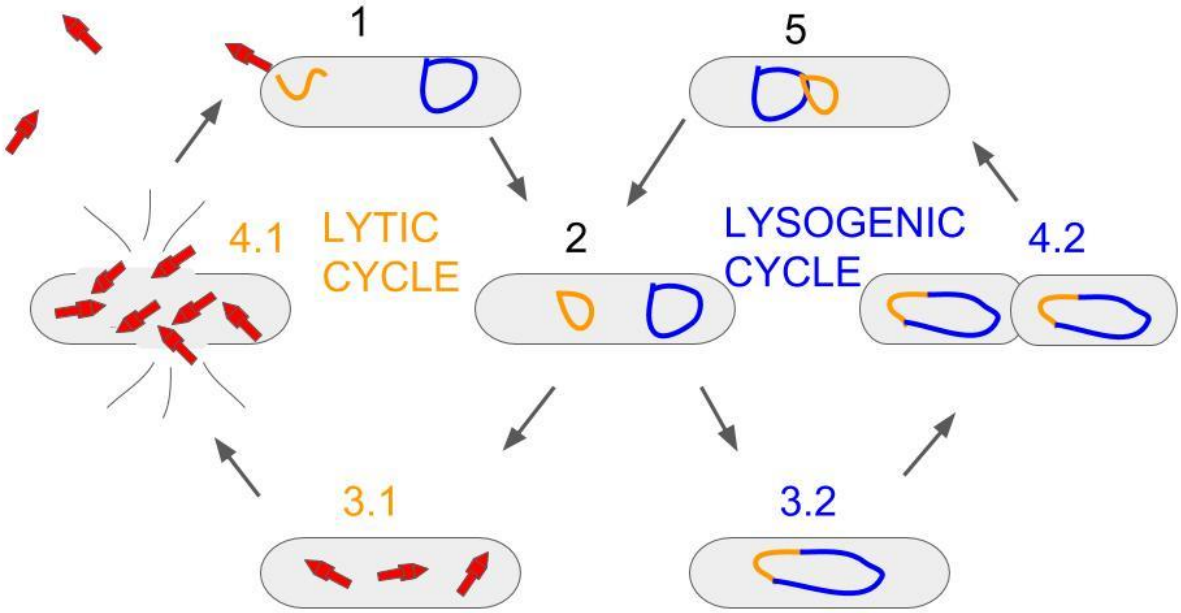

Figure 1. Differences between the lytic and lysogenic cycle for phage infection of bacteria. 1: Insertion of phage DNA, 2: Phage DNA circularizes. Next, the phage will cause a lytic or lysogenic infection. For lytic cycle, 3.1 shows progeny virus manufacture and 4.1 lysis. For lysogenic cycle, 3.2 depicts viral insertion of DNA forming a prophage, and 4.2 replication of DNA copying the prophage as well, 5 depicts a prophage excising from the bacterial genome.

Early Phage History

Phage therapy is as contentious a subject today as it was almost one hundred years ago when the first clinical study of phage therapy was published in 1921. This involved the direct injection of phage into staphylococcal boils (Deresinski, 2009). Some believe that the first example of phage therapy occurred in 1919 when an oral lysate was used to treat dysentery (Gill, Franco, & Hancock, 2015; Kutter et al., 2010; Wittebole et al., 2013). Many of these reports were not respected by the wider scientific community as the investigators failed to use good experimental technique with double-blind controls and many such "trials" were simply reports of a single clinician's administration of phage to treat a disease. Early phage therapy treatments were noted for their frequent clinical inconsistencies between different clinicians and scientists across borders. It is believed that lack of quality control and the technical expertise to prepare

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stable lysates were likely the main reasons why some clinicians were not able to replicate findings performed by other groups halfway around the world in early phage therapy trials. Often, investigators would find themselves obstructed by language barriers, as microbiologists could be in Brazil attempting to exchange data and results with scientists in Egypt, England, or Germany. Of commercial production of lysates D'Herelle stated, "On the whole, none of the preparations on the market is capable of effecting recovery from infectious disease" (Dublanchet & Bourne, 2007; Henein, 2013). A major reason why phage lysates failed is likely due to this premature commercialization of products that were not ready for public use or proven for clinical efficacy.

While the United States had started a Food and Drug Administration (FDA) a decade before phage therapy, it was mostly concerned with branding issues and adulterated products in the food industry and the medical field was not highly regulated. It was not until 1938 that a thorough pre-market review measure was passed in the legislature and added significant regulatory powers to the FDA (Shulman, 2013). These early pre-market reviews from the regulations of 1938 are the backbone of current FDA clinical trials.

With the advent of administrable antimicrobials in the 1930s, the antimicrobial age was born (Kutter et al., 2010). So confident were clinicians in the 1970s and 1980s, that it was believed by many, that a post infectious disease state had been achieved (Henein, 2013). With the rise of antibiotics, phage grew out of favor with the Western world, as they appeared to be much less efficacious than antibiotics. Proponents of antibiotics and phage recognized that administering antibiotics kills commensals and pathogens alike (Kutter et al., 2010). As we look forward to today, it is clear than antibiotics have serious side effects much more substantial than the loss of beneficial intestinal commensals. Antibiotic overuse has led to widespread resistance.

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Phage therapy while dismissed a hundred years ago, is experiencing a remarkable return to clinics, scientific benches, and regulatory awareness (Brüssow, 2012; Deresinski, 2009; McCallin et al., 2013; Ozkan et al., 2016).

Phage Therapy East to West

Phage research was present in the US in the 20th century, but it was an incredibly specific niche and an understudied scientific expertise. However, in the East, within Georgia, India, Russia, and to some extent France, phage work was much more well regarded, accepted, and practiced (Kutter et al., 2010; Sybesma et al., 2016). The Eliava Institute in Georgia has, for decades, kept a phage library of different cocktails of phage that can be used to treat a myriad of diseases. Libraries such as this have been essential in jumpstarting research into this area in the West. Clinicians can order specific phage for scientific investigation and occasionally U.S. doctors have prescribed phage. In addition, medical tourism is promoted by a California company to bring US, Australian, and European citizens to the Republic of Georgia for phage treatment (Kutter et al., 2010). Another institute that perhaps rivals Eliava is the Hirszfeld Institute in Poland. The institute has developed a world renown reputation for treating urinary tract infections, septicemia, *Shigella* infections, and various wound infections, including furunculosis (Kutter et al., 2010; Sulakvelidze, 2001).

Libraries such as those kept at the Eliava Institute will be essential for later regulatory frameworks. Efficacious cocktails could be created from phage in large phage libraries, where all phage deposited had passed regulatory approval (Mirzaei & Nilsson, 2015). In addition to "simple" product to market regulation from the FDA, there is little (Sulakvelidze, 2001) or no insurance coverage for phage treatment. It will be essential to prove to global insurance exchanges and national insurance companies that phage treatment is an acceptable course of

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treatment on its own, or as an adjunct therapy (Gill et al., 2015; Torres-Barceló & Hochberg, 2016a). Phage therapy may work synergistically with antibiotics. It is believed that as the bacteria respond to an antibiotic, they may upregulate phage receptors, and when bacteria respond to phage, efflux pump action may be downregulated (Torres-Barceló & Hochberg, 2016a).

Currently, the regulatory framework around phage use in the US is still being formed (Bassetti et al., 2017; Chan, Abedon, & Loc-Carrillo, 2013; Henein, 2013). The sooner the private sector, academia, and government entities can work together on this issue, the sooner phage therapy may be employed to help a worldwide infectious disease problem. In the developed world (US and EU), 50,000 citizens die each year from antibiotic-resistant infections (Torres-Barceló & Hochberg, 2016a). Bringing a new drug to market now costs between $400-800 million and can go as high as $1 billion. Since phage are so new to the regulatory scene, especially in the West, it is unclear if the cost will be lower and how the phage lysates will be protected by patents (Henein, 2013). Many questions remain as to how and when phage therapy will become a clinical reality, but it is clear that much effort remains in boosting public awareness, performing private research, and defining overall regulations.

Phage Therapy Successes

Phage therapy success can be seen by the increased phage vision that informed scientific investigators, public health officials, government regulators, and private parties now hold in comparison to two decades ago. With new challenges, such as antibiotic resistant bacteria, come new solutions and new opportunities. Phage therapy would probably not be considered in the current environment, were it not for the modern infectious disease crisis. The first double-blind experiment with phage only just occurred in the US less than a decade ago (Rhoads, 2009;

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Wittebole et al., 2013). The use of phage to decrease food-borne illnesses in ready-to-eat food preparations have been approved by FDA and used to prevent illnesses caused by *Listeria monocytogenes* and *Salmonella* (Henein, 2013; Torres-Barceló & Hochberg, 2016a). Current research is expanding towards the use of phage to treat fresh produce that may be contaminated with *E. coli* as well (Snyder, Perry, & Yousef, 2016).

Phage therapy would be especially important if it could be used to curb infections caused by multi-drug resistant strains of bacteria that boast high mortality rates such as carbapenem-resistant *Enterobacteriaceae* (CRE), *P. aeruginosa* and *S. aureus*. Research is ongoing but there is potential for both clinicians and patients to be optimistic, as many phage-resistant strains are susceptible to antibiotics and many antibiotic-resistant bacteria are susceptible to phage. It appears that the fitness of bacteria may be decreased when they are fighting a "two-front" war against multiple phage and multiple antibiotics. The susceptibility profiles of bacteria to phage and antibiotics are unrelated. Most antibiotic-resistant strains have been shown to be highly susceptible to phage. (Allen, Trachsel, Looft, & Casey, 2014; Ozkan et al., 2016; Torres-Barceló & Hochberg, 2016a).

As phage possess incredible specificity to their host, phage therapy is only possible if you have a phage specific to your target bacterium. With ingenuity and imagination, many diseases that have a clear etiology will be helped by increased phage work and phage awareness. A new "golden age" of microbiology may ensue as phage specific for known disease agents are discovered.

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Anthrax

History of anthrax

Anthrax has been feared for centuries, from Roman poets (Virgil) to Egyptian Pharaohs (Akhenaten) to ancient medieval monks and 19th century English drovers and wool workers (Monds & O'Toole, 2009; Schwartz, 2009; Van Ert et al., 2007). The same strains that might have been involved in Exodus chapter nine, that caused boils and death of livestock, could still be around today, as spores are resistant to heat, desiccation, UV light, gamma radiation, and many disinfectants (Sternbach, 2003). Anthrax is generally a disease of livestock, but much of human existence was spent in close proximity to animals and animal products, and exposure often led to cutaneous anthrax, or gastrointestinal anthrax if contaminated meat was ingested. Anthrax did not die out in the industrial age, but became a serious concern for the wool industry, so much so that pulmonary anthrax was termed "woolsorters disease" (Pilo & Frey, 2011; Schofield et al., 2013). Anthrax exposure is still an occupational hazard for agricultural workers in developing countries, tanners, laboratory workers, and soldiers (Craft, Lee, & Rowlinson, 2014; Lekota et al., 2016). Currently, anthrax is not endemic in most of the developed world due to early large-scale control efforts. However, anthrax is endemic for much of South and Central Asia, Afghanistan, Bangladesh, India, Iran, Pakistan, Kazakhstan, Kyrgzstan, Turkey, and the Republic of Georgia (Price et al., 2012).

Discovery of anthrax

Robert Koch is credited with the discovery of the cause of anthrax in 1876, showing definitively through his postulates that the disease was caused by a single etiological agent. Koch worked with *B. anthracis* for much of his career, and made discoveries in the life cycle and sporulation of this organism (Gillis & Mahillon, 2014). A research rivalry developed between

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Koch in Germany and Louis Pasteur in France. Pasteur extended animal experiments, definitively illustrating that immunity could be induced with a live attenuated vaccine (Fouet, 2001). Pasteur even hypothesized that *B. anthracis* could be carried by earthworms, an astute observation with the little resources he had. Earthworms have since been implicated as environmental reservoirs of *B. anthracis* (Schuch & Fischetti, 2009).

Morphology

B. anthracis can form an especially robust endospore and like most spore formers, is gram positive. *B. anthracis* is non-motile, non-hemolytic, negative for phospholipase C production, and sensitive to the gamma phage and to penicillin (Klee et al., 2006; Rao, Mohan, & Atreya, 2010). Colony morphology and capsule staining, in addition to the other factors listed, are tests that can help distinguish *B. anthracis* from other *Bacillus* species. (Pilo & Frey, 2011) *B. anthracis* is also closely related to *B. thuringiensis,* notable for its use as a natural insecticide, and *B. cereus* which is a common cause of human food poisoning. *B. anthracis, B. cereus,* and *B. thuringiensis* are members of a unique clade. Of these three closely related strains, *B. anthracis* is the most monophyletic (P Keim et al., 2000, 2000; Wang & Ash, 2015). Despite similar genetics, the phylogenetics and pathogenicity do differ among sister strains.

Sporulation

B. anthracis is monophyletic because rather than existing as a constantly replicating vegetative cell, it is found mostly as a dormant spore. Since a spore is metabolically inert, the organism does not mutate at the same rates of other species (Paul Keim et al., 2004). The spore is extremely hardy, even when compared to other endospores, as it also has an extra layer termed the exosporium. However, *B. anthracis* cells are less hardy overall than other *Bacillus* species as their vegetative state is unstable and survives poorly in the environment without the help of

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lysogenic phage which affect sporulation, growth in worms, and growth in the wider environment (Ganz et al., 2014; Gillis & Mahillon, 2014; Koehler, 2009; Schuch & Fischetti, 2009). *B. anthracis* spores are about 1 µM in diameter, and once ingested by macrophages, can remain dormant for indefinite periods of time; a hundred days or more has been seen in human primate studies. For recent outbreaks such as the Sverdlovsk incident in Russia in 1979, 2-43 days was the time range between infection and appearance of symptoms (Toole et al., 2002).

Pathogenicity of anthrax

Anthrax is transmitted by three routes, causing the following types of diseases: 1. Cutaneous, 2. Gastrointestinal, and 3. Pulmonary or inhalational. A fourth route is markedly more severe than cutaneous anthrax, and is termed injection anthrax, occurring mostly in drug users employing needles. This is a new public health hazard and the term has been employed for less than two decades due to differential changes in drug use across the globe. It was determined that the heroin supply was being diluted with crushed bone (likely from infected animals) in Afghanistan. As Afghanistan produces over 90% of the world's heroin, it soon became a global public health crisis (Price et al., 2012).

B. anthracis is only pathogenic if it has both virulence plasmids. Plasmid pX01 (174 kbp) encodes for the toxin genes (peg, lef, cya) and plasmid pX02 (95 kbp) holds the genetic material for capsule synthesis (capA, capB, capC) (Paul Keim et al., 1997; Pilo & Frey, 2011). The *B. anthracis* Sterne strain is non-pathogenic as it does not have the pX02 plasmid. Without this plasmid, the Sterne strain cannot produce the capsule and therefore is not pathogenic (Cataldi, Mock, & Bentancor, 2000).

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Symptoms and Treatment

Early infection with *B. anthracis* will cause overall malaise at first, while the organism is setting up a systemic infection and shutting down the innate immune system. Symptoms may include coughing, headache, vomiting, chills, weakness, and abdominal pain. In late stages of infection, which occur suddenly, fever, sweating, and shock can quickly ensue with pronounced lymphadenopathy and possible hypoxia. The cardiovascular system and liver are prominently targeted by the toxins which can cause organ failure and death. Fifty percent of patients may also exhibit symptoms of meningitis. (Liu, Moayeri, & Leppla, 2014; Toole et al., 2002). Treatment of anthrax with antibiotics such as, ciprofloxacin, vancomycin, penicillin, doxycycline, or erythromycin, will kill the bacteria, but it will not stop toxins already present in the body so death can still ensue. Current research is investigating small interfering molecules that may block some of the harmful effects of the lethal factor (LF) and edema factor (EF) toxin moieties (Liu et al., 2014). As some penicillin-resistant strains of *B. anthracis* have been found in nature, ciprofloxacin is the preferred treatment (Bossi et al., 2006).

Anthrax in Bioterrorism and Biowarfare

Anthrax was a major concern for the middle part of the 20th century. England, Russia, Germany, Japan, and the United States all had thriving bioweapon research programs. An anthrax attack for bioterrorism is believed to be a credible and deadly threat to national security. Anthrax spores are very hardy and relatively easy to manufacture, mill, and statically charge for weaponization.

It is believed that 50 kg of concentrated *B. anthracis* spores released over a city of 5 million would kill 100,000 and sicken a quarter million, and under ideal weather conditions, these numbers could increase by one log. If the payload was doubled to 100kg, the death toll

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would equal that of a hydrogen bomb. (Bossi et al., 2006; Toole et al., 2002). This would also cause massive public panic and shut down many public and private services. Bioterrorism can produce huge casualties, but it is also a type of psychological warfare, in that it can be used to break a nation's trust in the systems that they rely upon for daily life such as transportation networks, water systems, and hospitals. Inhalational anthrax can be infectious with anywhere from 8-50,000 spores. The LD50 for these spores is estimated to be between 2,500 and 50,000. Many people may require much less, as primate data suggests a mere 1-3 spores is capable of causing serious infection (Toole et al., 2002).

Bioterrorism is not to be dreaded every day, but nations should be prepared. As the 20th century came to a close, few large-scale bioterrorism attacks had been committed in the public's recent short-term memory. The bioterror threat was felt to be overstated and emergency preparedness budgets were not popular and perennially cut (Craft et al., 2014). The 2001 anthrax letter attacks increased federal funding substantially, but this has mostly disappeared as no new attacks have been reported (Gillis & Mahillon, 2014; Van Ert et al., 2007). A new challenge has awakened in America with overregulation of scientific laboratories dealing with BSL-3 agents. Due to the increased reporting, training, and cost of running these laboratories, many labs are not currently situated to do BSL-3 work. When the barriers to entry for BSL-3 work are too great, it places individuals and nations at risk. When we overprotect negligent agent release by excessive and burdensome barriers, the only thing that happens is less research that can help mitigate the effects of the next bioterrorism event (Wurtz, Grobusch, & Raoult, 2014). All types of research that are not offensive bioterrorism research, should be allowed and encouraged without overburdening regulation, including phage therapy (Barras & Greub, 2014; Riedel, 2004).

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INTRODUCTION

B. anthracis phage therapy

Known phage infecting B. anthracis

Within a decade of the introduction of phage therapy in 1919, work was performed by D'Herelle in 1929 to combat anthrax in mice with phage (Walther, 2003). This work, although laudable, yielded no scientific record of clinical significance. Pasteur had already shown how to immunize cows against anthrax with a live vaccine decades earlier (Letarov, Biryukova, Epremyan, Shevelev, & Letarov, 2016). In 1951, phage typing started with the isolation of the *Bacillus* phage gamma. The gamma phage was highly specific with only a few strains lysed that were not *B. anthracis.* Notable exceptions to this rule were *B. cereus*, isolates ATCC 4342 and NCTC 1651, which the gamma phage did successfully infect. (Sozhamannan et al., 2008). In addition, the gamma phage, while lysing some *B. cereus* strains, was also unable to lyse some very specific *B. anthracis* strains. The gamma phage was used as one of the many diagnostics to identify *B. anthracis* and is believed to be over 95% accurate as a diagnostic test, which is above United States Army Medical Research Institute of Infectious Disease (USAMRIID) requirements (Abshire, Brown, & Ezzell, 2005).

The protein conferring lysis to the gamma phage was termed PlyG. Later, another lysogenic phage Ba02 yielded another lysin termed PlyL. PlyL and PlyG endolysins have two domains: a cleavage domain and a recognition domain. It is hypothesized that both endolysin's recognition domains attach to molecules covalently bound to peptidoglycan (Ganguly et al., 2013). The gamma phage is a *Siphoviridae* virus as it is a tailed phage with a non-contractile tail (Morimoto;Shiomi, 1975). In the former Soviet Union, another phage, bacteriophage Fah, was used as a diagnostic tool in place of the gamma phage that is so well characterized in Western

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scientific literature. Fah is strictly lytic and its genetic origins are unknown. Prophage similar to it are found in isolates around the world and it is unclear whether the modern Fah bacteriophage is the parent or daughter to the lysogenic phage. Fah was still produced into the 20th century, but it is unknown whether it is still produced for veterinary and laboratory uses today (Minakhin et al., 2005). While the host range of Fah is unknown, at least to public databases, recently isolated phage has demonstrated better discrimination than the gamma phage. A recent phage, AP50, was able to lyse 111/115 *B. anthracis* strains (97%) and none of the 100 *B. cereus* strains, while the gamma phage lysed 105/111 *B. anthracis* strains and is known to infect certain *B. cereus strains*. It appears that although gamma is the oldest diagnostic phage, it may not be the best suited for clinical testing (Sozhamannan et al., 2008).

In addition to the well-known Ba02 lysogenic phage, there are multiple phage that are highly conserved in the majority of *B. anthracis* strains (Ganz et al., 2014; Gillis & Mahillon, 2014). These prophages and one non-sense mutation are what characterize *B. anthracis f*rom many other closely related strains of *Bacillus,* especially the closely related *B. cereus* and *B. thuringiensis.* While *B. anthracis* is noted for having a short vegetative state, it is hypothesized that prophage help the host's sporulation, vegetative soil lifecycle, infection of earthworms, and overall fitness. As many as sixteen prophage may be found in *B. anthracis,* with other investigations only counting seven *Siphoviridae* prophage and eight *Siphoviridae* lytic phage. Other phage isolates have been found and more will likely be found in the future, as three new *B. anthracis* phage were recently found in 2017, and every few years new phage for this species are isolated (Alkalay, Sternberg, Coppenhagen-Glazer, & Hazan, n.d.; Ganz et al., 2014; Gillis & Mahillon, 2014; Schuch & Fischetti, 2009).

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Adjunct therapy potential and possible concerns

Phage combined with antibiotics have the potential to be a cleaner, safer, cheaper, more efficacious, and more specific alternative to simple antibiotic treatment alone. Phage and antibiotics combined could be a powerful clinical tool. It could be postulated that if *B. anthracis* phage were to work well as therapeutics, it would have already been low hanging fruit and completed, but this is not the case as *B. anthracis* phage work has lagged behind that of most other bacterial genera.

Phage are a more natural alternative therapy and may be a good option for those who are reluctant to use traditional medications and drugs. Phage are cheaper as they are easily produced and increased dosage does not always mean a drastic increase in cost (Ozkan et al., 2016). They are more efficacious when used together with antibiotics than antibiotics alone, as the activity of antibiotic resistance is separate from phage resistance and antibiotics and phage have distinct modes of action (Gill et al., 2015; Qadir, 2015; Torres-Barceló & Hochberg, 2016b). Phage are highly specific and are self-limiting; if the host is not present then neither will be the phage.

The self-limiting aspect of phage and the broad-spectrum aspect of antibiotics are essential distinctions between phage and antibiotics. Antibiotics can provide a possible clearance of infectious disease with unclear etiology, while phage have the unique specificity to treat an infection and not destroy commensal flora (Chan et al., 2013). A myriad of concerns about bacteriophage containing toxins or acting as nanoparticles with surface charges and possible aggregation issues have been voiced. These concerns are less important when viewed in light of how phage can be treated as nanoparticles in safety studies, how toxins can be screened, and the fact that phage have been administered in many different ways at varying dosages for nearly a century with little or no ill effects (Rhoads, 2009).

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The safety of phage will only increase with increased regulation, awareness, and phage toxin testing. The benefits appear to outweigh any potential costs that are currently envisioned (Deresinski, 2009; Endersen et al., 2014; Sulakvelidze, 2001). Any concerns about safety can be worked through, and it appears that overall, phage administration is much safer and causes less harm to patients than antibiotics. Alexandra Henein poses an intriguing question, "Considering reports that phage therapy has been widely successful even when used as a last resort in infections non-responsive to antibiotics, a question arises: Is it even ethical to continue not pursuing phage therapy?" (Henein, 2013). Regulatory issues will likely be worked through as phage trials show successes. Phage resistance will occur and may be overcome as more novel phage are found and new cocktails pass safety concerns. Existing phage can likely be manipulated in the lab to increase their host range or include novel endolysin genes. Although it may appear to outsiders that novel scientific discoveries are decreasing with each passing year, this could not be further from the truth. "Even as biodiversity seems to be everywhere under threat or in retreat, scientists are discovering and naming new species at a greater rate than anytime during taxonomy's 250-year history" (Stutz, n.d.). This is true for plants, animals, and microbial life---especially for phage.

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MATERIALS AND METHODS

Choosing pathogenic strains for testing

Importance of genetic profile

With around 89 strains of *B. anthracis* that are commonly considered the worldwide type strains, and thousands more clinical isolates, it is difficult to choose a proper battery of strains for testing (P Keim et al., 2000). It was imperative to choose a number of strains for testing that would represent the extant genetic diversity of pathogenic *B. anthracis*, while also being manageable for rigorous testing and regulatory compliance in the BSL-3 research environment (Wurtz et al., 2014). The BYU Select Agent Archive has over 300 strains, and 88 of the 89 type strains at the writing of this paper. Performing tests on eighty-eight of the 300 strains was not technically viable and many of the results would likely be similar, as *B. anthracis* is relatively genetically homogenous, even compared to other *Bacillus* species.

Justification of Selected Strains

In order for our investigations to be easily replicated, we used only type strains and no clinical isolates. *B. anthracis* is grouped into three different branches, or groups: A branch, B branch, and C branch (see Figure 2 and Table 1). All of our testing was done with isolates from the A and B branches, with 11 distinct pathogenic strains, and with the non-pathogenic *B. anthracis* Sterne strain, for a total of 12 diverse strains. Testing of a strain that represented the C branch was not possible in time for this work, but it will be evaluated later in further investigations that are already planned. The eleven pathogenic strains that were used in this work are well known to represent the extant genetic diversity of *B. anthracis* in the A and B branches (Pilo & Frey, 2011; Van Ert et al., 2007).

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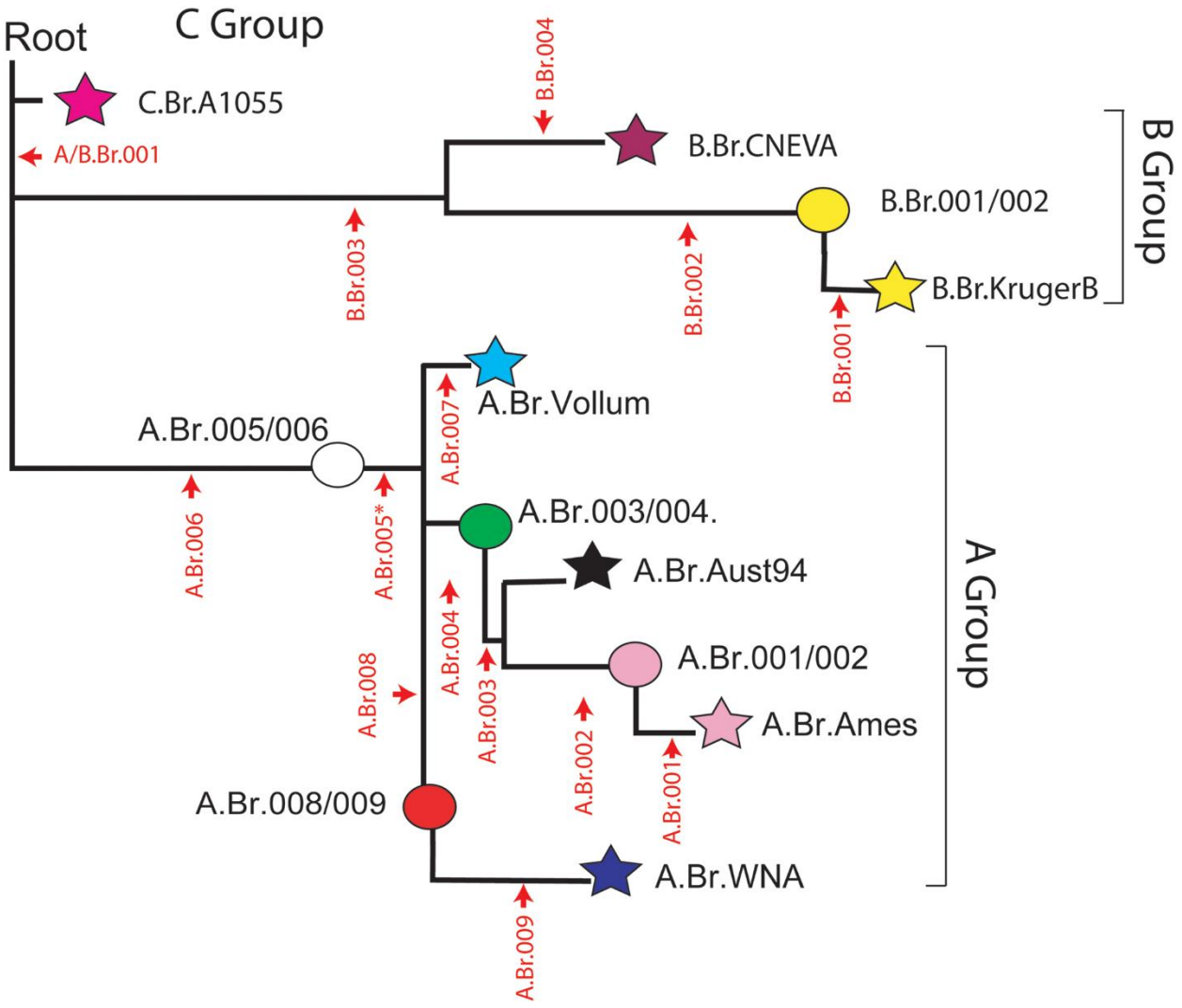

Figure 2. Figure and data by Van Ert et al. showing the genetic tree of the A and B groups tested. One C group strain is also shown. Stars and circles represent strains and red arrows designate strain relatedness. The genetic study was performed through SNP typing.

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Table 1. Table and data by Van Ert et al. showing the SNPs between the various strains. The Lineage/Group column and Type Strain column correlate with circles and stars in *Figure 2,* which represent the type strains used in our tests.

Lineage/Group	Type Strain.	Sequence	A.Br.001	A.Br.002	A.Br.003	A.Br.004	A.Br.006	A.Br.007	A.Br.008	A.Br.009	B.Br.001	B.Br.002	B.Br.003	B.Br.004	A/B.Br.001
C.Br.A1055	C.A1055	C.USA.A1055	T	G	A	T	C	T	T	A	T	G	G	T	**G**
B.Br.KrugerB	B1.A0442	KrugerB	T	G	A	T	C	T	T	A	**C**	T	A	T	A
B.Br.001/002	B1.A0102		T	G	A	T	C	T	T	A	**T**	**T**	A	T	A
B.Br.CNEVA	B2.A0402	CNEVA.9066	T	G	A	T	C	T	T	A	T	G	A	**C**	A
A.Br.Ames	A2.A0462	Ames	**C**	A	G	C	A	T	T	A	T	G	G	T	A
A.Br.001/002	A2.A0034		**T**	**A**	G	C	A	T	T	A	T	G	G	T	A
A.Br.Aust94	A1.A0039	Australia94	T	**G**	**G**	C	A	T	T	A	T	G	G	T	A
A.Br.003/004	A2.A0489		T	G	**A**	**C**	A	T	T	A	T	G	G	T	A
A.Br.Vollum	A1.A0488	Vollum	T	G	A	T	A	**C**	T	A	T	G	G	T	A
A.Br.005/006	A1.A0158		T	G	A	**T**	**A**	**T**	T	A	T	G	G	T	A
A.Br.008/009	A1.A0293		T	G	A	T	A	T	**G**	**A**	T	G	G	T	A
A.Br.WNA	A1.A0193	W. N. America	T	G	A	T	A	T	G	**G**	T	G	G	T	A

CanSNPs and profiles for the lineages/groups: This table lists each of the 12 lineages and groups and indicates the canonical SNPs that help to define each of the sub-lineages and sub-groups (canSNPs that define a particular sub-lineage or sub-group are indicated in yellow). Each lineage is named after the whole genome sequence that is positioned as an end point in a branch created by a comparison of that particular genome sequence to 6 other genomes (stars in Figures 1 and 3). As endpoints all but one of the lineages are defined by a single canSNP (see profiles in yellow for B.Br.Kruger , B.Br.CNEVA, A.Br.Vollum, A.Br.Ames and A.Br.WNA. Although Aust94 is an endpoint the canSNPs that define this lineage were developed before the draft sequence and as a result two canSNPs A.Br.002 and A.Br.003 define the branch point where this isolate is located. Similarly, the groups are positions that define branch points [5,35] along the different lineages (Circles in Fig. 1 and 3). They carry the group name designations corresponding to the canSNPs that flank these positions and are indicated in blue in this table (e.g. A.Br.001/002). Note that the sub-group need at least two canSNPs (one SNP on either side of the node) to assign a correct sub-group. Sub-group A.Br.005/006 requires three canSNPs to assign an exact genotype because a canSNP for A.Br.005 has not yet been tested. The whole genome sequences for Bacillus anthracis strains A0155, Ames Ancestor, CNEVA-9066, Kruger B, Vollum, Western North America (WNA) and Australia 94 can be found in the NCBI microbial genome website at http://www.ncbi.nlm.nih.gov
doi:10.1371/journal.pone.0000461.t001

Phage isolation and screening

Sampling

Soil samples were taken across three states in the Western United States: Idaho, Utah, and Nevada. All samples were fine soil samples except for sample 16, which consisted mostly of wood chips. Samples came from rural, urban, and suburban environments. A description of these samples and their locations is shown in Table 2 and Figure 3. Soil was taken beneath the grass line and any foreign objects removed. Soil sample size was approximately 10g and these were stored in sealed plastic bags without temperature control. After transportation to the lab, samples were kept at ambient temperature in lab drawers until processed.

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Table 2. Names and locations of the various soil samples used in this study. The number in the sample # column was used as an identifier during spot testing in the BSL-3 laboratory. The sample name also corresponds to the phage names.

Sample #	Name of Sample	Location of Sample
1	R House 2	Elko, NV
2	RC2	Elko, NV
3	MILO	Rexburg, ID
4	Timpview	Provo, UT
5	GMA House	Lehi, UT
6	Moms Garden	Rexburg, ID
7	Lehi Park	Lehi, UT
8	Backyard GMA	Lehi, UT
9	Gutter	Provo, UT
10	Kate 2	Lamoille, NV
11	Duck Pond	Provo, UT
12	Ryan's Corner	Provo, UT
13	Harvey Fletcher	Provo, UT
14	Rock Canyon	Provo, UT
15	FOB	Provo, UT
16	Temple	Provo, UT
17	Wayne	Lamoille, NV
18	Elko Tree	Elko, NV
19	Nate2	Lamoille, NV
20	4NSE	Provo, UT
21	Lacey	Rexburg, UT
22	Elko Garden	Elko, NV
23	Marb	Provo, UT

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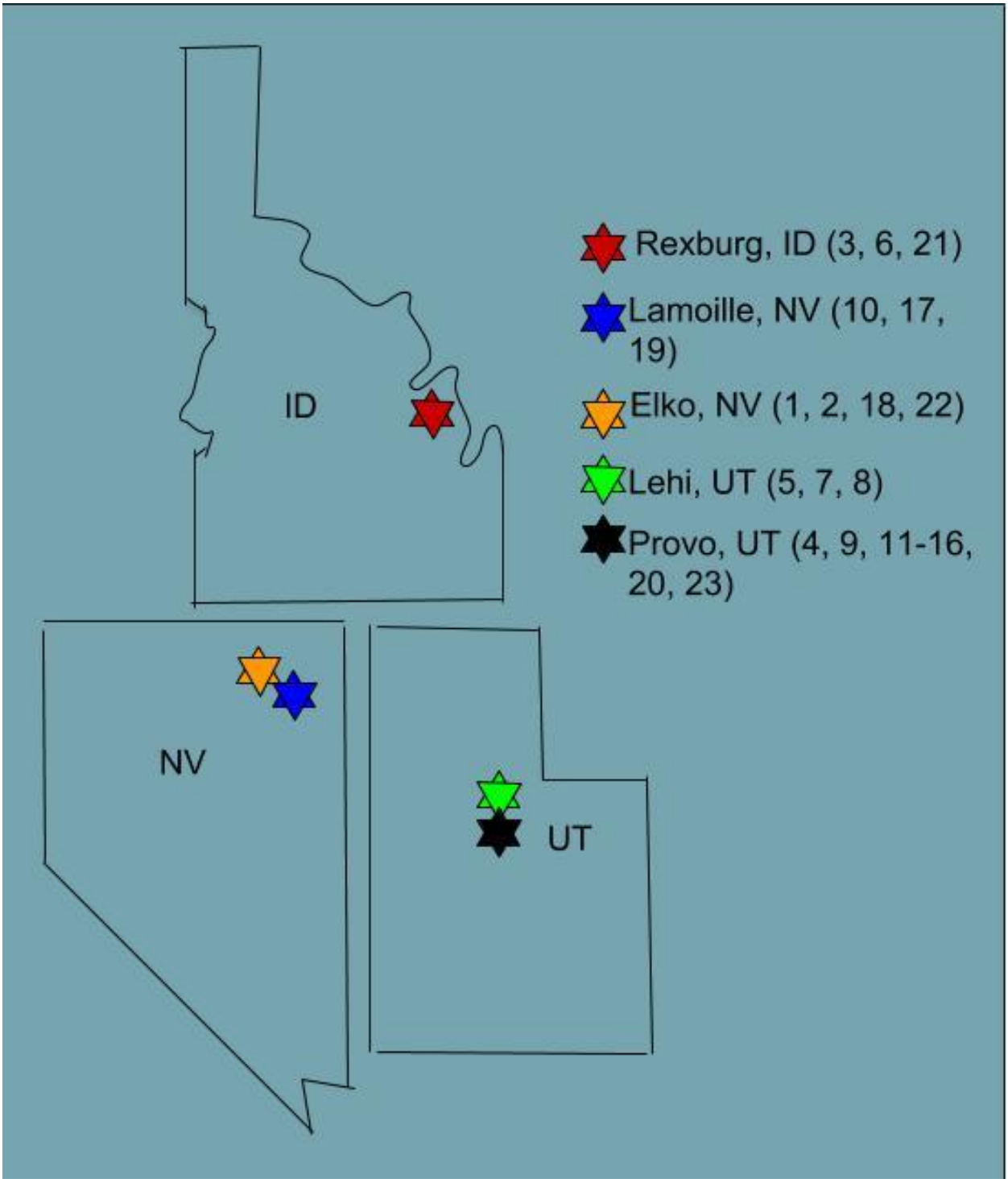

Figure 3. The approximate locations where each soil sample was taken. Phage isolated from each location are listed in parentheses. Stars represent different cities or towns where samples were taken. RED, Rexburg. ORANGE, Elko. BLUE, Lamoille. GREEN, Lehi. BLACK, Provo.

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Assays using the Sterne strain

Enrichment Culture:

The following procedure was used to enrich for *B. anthracis*-specific phage:

1. Add 1ml *B. anthracis* Sterne (overnight culture) to 150ml flask
2. Add 10 ml LB broth to flask
3. Add 1g soil sample to flask
4. Incubate at 30 degrees Celsius for 2 days in shaking incubator

Filter and Plate enrichment culture:

The following procedure was used to visualize *B. anthracis*-specific phage:

1. 10 ml of enrichment culture was placed in a 15ml tube
2. Tube was centrifuged for 50 min at 4,000 rpm
3. Filter sample with .45um filter and syringe into new 15ml tube
4. Put 0.5ml *B. anthracis* Sterne culture in a red cap tube
5. Infect with 5 ul of filtered enrichment suspension
6. Incubate for 45 min at room temp
7. Plate with 5ml top agar
8. Let set (approximately 2 hours)
9. Place in 30-degree Celsius incubator for 48 hours

Plaque Purification Protocol:

The following procedure was used to purify each *B. anthracis*-specific phage:

1. Put 200 ul LB broth in a microcentrifuge tube
2. Pick plaque with pipette tip
3. Place pipette tip in the microcentrifuge tube with broth and mix

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4. Put 0.5ml *B. anthracis* Sterne culture in a red cap tube
5. Infect with 50 ul of plaque solution
6. Incubate for 45 min at room temp
7. Plate with 5ml top agar
8. Let set (approximately 2 hours)
9. Place in 30 degree Celsius incubator for 48 hours

High titer Lysate Protocol:

The following procedure was used to make and verify a high titer lysate for *B. anthracis*-specific phage:

1. Put 1 ml *B. anthracis* Sterne culture in a 150 ml flask with 10 ml LB
2. Pick plaque from plaque purification plate with pipette tip
3. Infect *B. anthracis* in flask with the plaque
4. Incubate for 2 days at 30 degrees Celsius in shaking incubator
5. Centrifuge and Plate High Titer Lysate:
6. Put 10 ml of high-titer lysate (HTL) in 15ml centrifuge tube
7. Centrifuge for 50 min at 4,000 rpm
8. Filter with 0.45um filter and syringe
9. Put 180 ul LB broth in 3 micro centrifuge tubes
10. Do serial dilution starting with 20 ul filtered HTL (10 fold dilutions)
11. Plate dilutions 1, 2, and 3 with 5 ml top agar
12. Let set for 2 hours
13. Place in 30-degree Celsius incubator for 48 hours
14. Count plaques on plate for titer

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Phage Digest

In order to show genetic differences of the phage, a restriction enzyme digest was performed. The restriction enzyme *Hind*III was used according to the following procedure:

1. Add 2 microliters of CutSmart and 17 microliters of DNA to a microcentrifuge tube and mix well
2. Add 1 microliter of *Hind*III enzyme
3. Incubate for 1 hour at 37 degrees Celsius
4. Run digest on 1% agarose gel for 40 min at 150V
5. Visualize gel

Electron Microscopy

Bacteriophages are the most abundant biological entity on the planet and likely contaminate many electron microscopy (EM) samples by accident. In our work, we were trying to visualize only bacteriophage. Once a phage had been properly isolated through plaque assays and a concentration of 10^7 plaque forming units (PFU) per milliliter had been obtained, electron microscope images were taken for classification and measurements. While the process of preparing a sample seems rather easy, if not done precisely, images will be low quality and not viewable. The steps in this process were as follows:

1. Measure 0.2 grams of uranyl acetate in a glass contained scale on a weigh boat
    a. Glass contained scale is used because of the danger of the uranyl acetate
2. Mix the uranyl acetate with 10 ml double distilled water in 15ml tube by shaking
    a. Mixing the powder with the water allows the uranyl acetate to become aqueous, which is necessary for this process

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b. Important: Steps 2-10 were done under a fume hood because uranyl acetate is a hazardous material

3. Pipette a dot of 10 microliters of uranyl acetate onto Parafilm using a p 20 pipette-man
4. Pipette a dot of 10 microliters of bacteriophage sample next to the uranyl acetate
5. Remove a single grid from the grid holder using tweezers. IMPORTANT: only touch the tweezers to the outer edge of the grid so the grid is not damaged

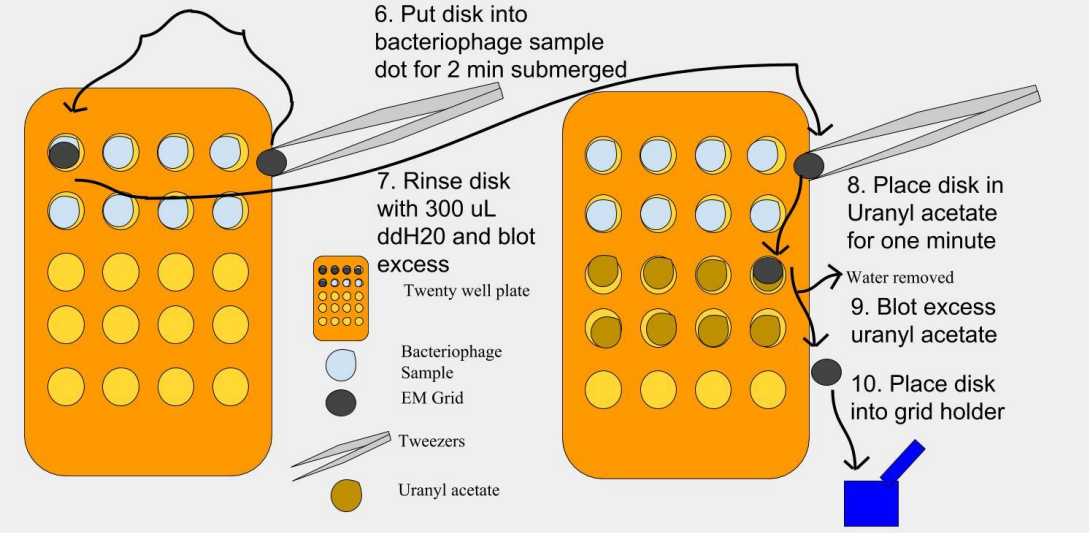

Figure 4. A simple schematic outlining steps 6-10 in the EM sample preparation protocol.

See Figure 4 for a schematic representing steps 6-10.

6. Place it in the bacteriophage sample dot for 2 minutes (completely submerged)
    a. This is done to allow the bacteriophages to remove themselves from solution and stick to the material on the grid
7. Take the grid out of the sample and run 300 microliters of double distilled water over the grid slowly using a p 1000 pipette-man, catching runoff with a paper towel. IMPORTANT: place the tip of the pipette close to the edge of the grid, but do not touch it to the grid

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a. This is done to take off any excess material and remove any film that attached to the grid

8. Place the grid in the uranyl acetate dot for 1 minute
    a. This allows the uranyl acetate to attach to the bacteriophage, allowing for visualization in the microscope (it acts as a negative stain)
9. Take the grid out of the uranyl acetate dot and blot the excess by lightly touching the edge of the grid to a paper towel
    a. This allows excess uranyl acetate to be removed, thus providing less exposure to hazardous material and a cleaner image
10. Place the grid back into the grid holder
    a. This allows one to keep track of the grid and not touch the samples, which could ruin them.
11. Write down which sample was put into which column on the grid holder.
    a. This allows the technician to connect the electron microscope image to the sample.
12. Take sample to Electron microscopy specialist to run samples. All samples were run on a Thermo Fisher Scientific Helios Nanolab 600 operated in STEM mode.

BSL-3 phage testing

Growing virulent B. anthracis strains

Virulent *B. anthracis* strains were grown in containment at all times per CDC regulations. Compliance to all policies and procedures concerning BSL-3 select agents was followed strictly. Strains were taken from freezer stocks and streaked for isolation using 10 µL sterile inoculation loops (Greiner Bio-One North American 200mm). LB agar plates were used. Following

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successful isolation of single colonies, stock plates were maintained at 4 degrees Celsius for 2 weeks. Isolates were grown at 37 degrees Celsius and double-bagged in plastic bags. Twelve hours yielded miniscule, but visible, colonies on LB agar. A full day's growth at 37 Celsius yielded larger colonies. All work was started with *B. anthracis* colonies that were incubated at least 18 hours.

Spot testing

Spot testing is an essential technique used to quickly and easily scan various phage against a single host strain. Most spot tests are later confirmed by plaque assay analysis, which is the gold standard for showing true phage infection. The steps for spot testing are outlined below:

1. Gather 11 strains of *B anthracis*: A34, A39, A102, A158, A193, A293, A402, A442, A462, A488, A489 and *B. anthracis* Sterne.
2. Grow *B anthracis* strains from an isoplate in a 50 mL conical tube overnight or a 250 mL glass flask. Place on shaker at 200 RPM overnight. If growing in a flask, use a cotton stopper to allow air access. Double bag the flask or conical tubes while still allowing passage of air.
3. Add 0.5 ml of overnight culture to a 15 mL conical tube
4. Add 5 mL of top agar (LB broth with agar)
5. Take mixture of top agar and bacteria and carefully pour onto petri dish partially filled with set agar
6. Wait a few minutes for the agar to set
7. Spot 2 10 uL drops of phage (23 distinct viruses were tested) onto a quarter of each plate. Each plate was typically inoculated with 8 drops.
8. Wait 12-24 hours and check plates for successful infection

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9. Analyze plates in hood by looking for clear plaques where spots were dropped
10. After recording data, dispose of plates in large ziplock bags. If culture was grown in a flask, sanitize with 3% peroxyacetic acid or 0.65% bleach and remove from hood and autoclave. If grown in 50 mL conical tube, tightly twist tube top and dispose in biohazard bin and autoclave.

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RESULTS

Phage Identification and Characterization

Testing phage on Sterne strain

A phage enrichment was performed for each of the twenty-three soil samples shown in Table 2 and Figure 3. After enrichment, the cultures were plated on *B anthracis* Sterne strain under BSL-2 conditions. Figure 5 shows multiple plaques on *B. anthracis* Sterne from the enrichment culture of the Wayne sample.

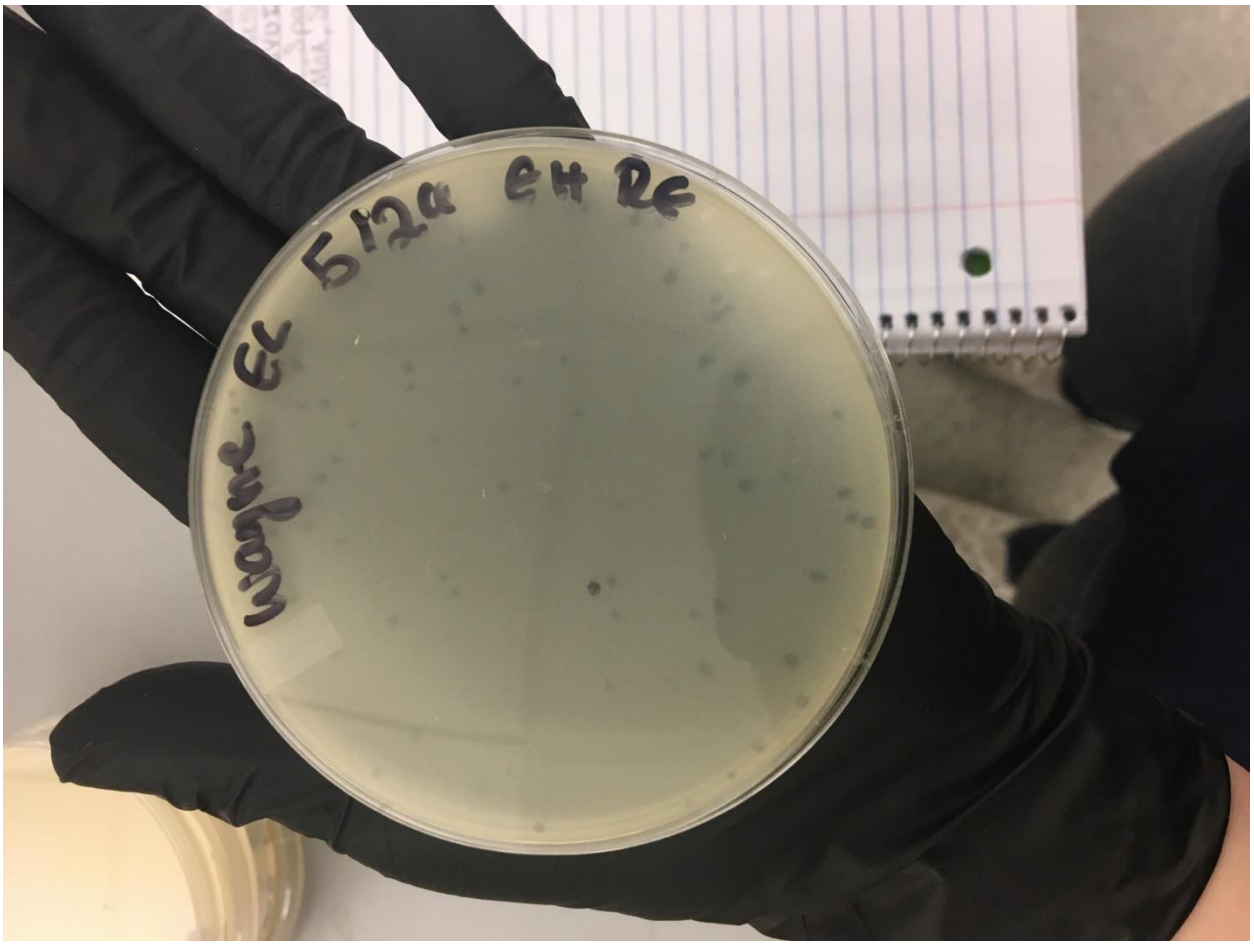

Figure 5. Phage plaques from the enrichment culture of the Wayne sample from Lamoille, NV grown on *B. anthracis* Sterne. The plaques shown were picked and re-isolated until plaque morphology was consistent.

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After successful infection from an enrichment culture, virus was purified by following the procedures in the plaque purification protocol in the Materials and Methods section. The plaques were then re-isolated until plaque morphology gave consistent and reliable results. After this purification step was completed several times, it was assumed that that the virus was monoclonal. Figure 6 illustrates an infection from a high titer lysate created from sample Elko Tree.

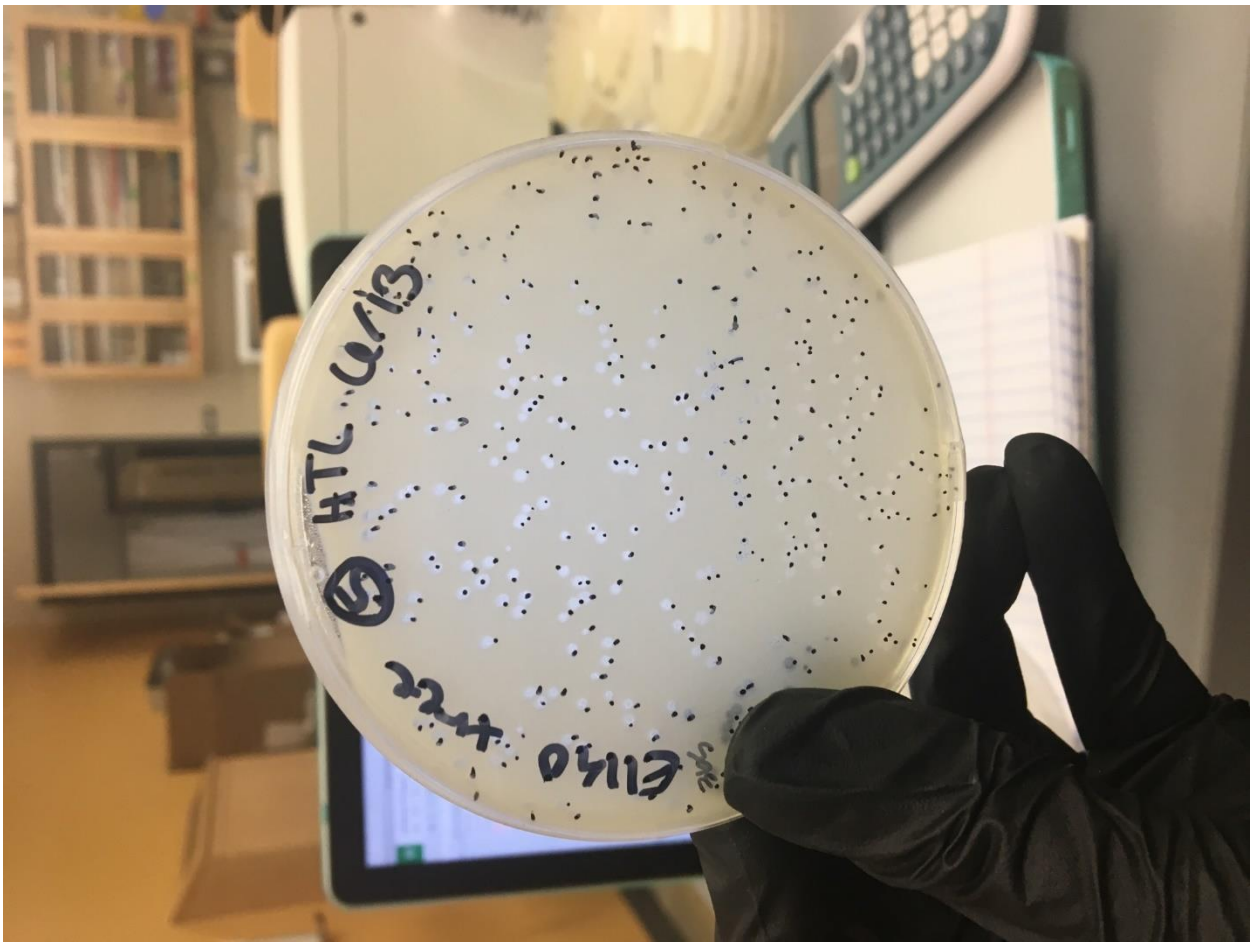

Figure 6. Successful infection of *Bacillus anthracis Sterne* from a purified phage isolate of Elko Tree.

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Intriguingly, despite the overall genetic similarity between various strains of *B. anthracis,* not all of the phage that infect a particular strain were able to be grown to the same titer. After a high titer lysate is achieved, a titer is performed to determine phage concentration in PFUs. Figure 7 shows the plating results from Step 11 of the high titer lysate protocol from Materials and Methods.

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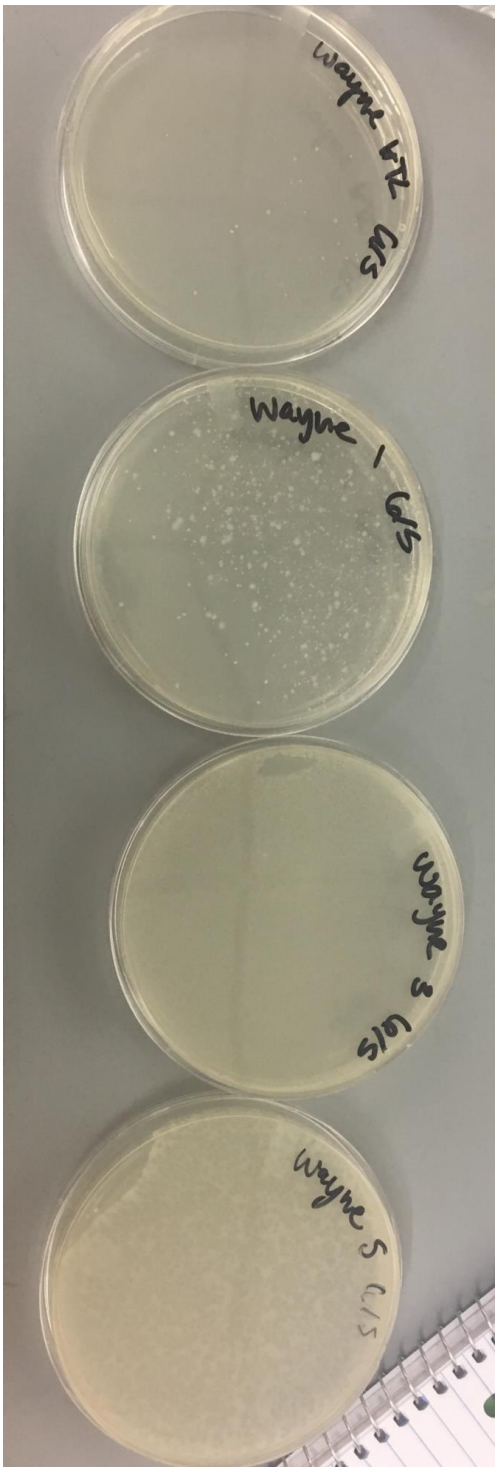

Figure 7. A phage titer as determined from plating serial dilutions of the high-titer phage suspension. Shown from top to bottom are plates of the undiluted lysate and dilutions: 10^-1, 10^-3, 10^-5. The colony growth shown on the undiluted and 10^-1 plates is likely rom germinated spores.

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Phage Differentiation

Phage isolation through BSL-2 testing on Sterne strain (Sampling, Isolation, Electron microscopy, DNA isolation and restriction digests) were performed by Emily Hansen and Rebecca Eardley (Grose Lab, Microbiology and Molecular Biology Department, BYU).

Genomic Digests

*Hind*III restriction digests were performed on DNA purified from each of the phage. Gels from these digests are shown in Figures 8 and 9. This was done to quickly determine which phage might be identical, so that duplicate sequencing could be avoided. From these investigations, most phage appeared genetically distinct from each other, with the exception of Ryans Corner and Harvey Fletcher depicted in Figure 9. There results will be confirmed by complete sequencing and annotations that will be performed later.

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Figure 8. A gel showing the *Hind*III digestions of various phage genomes isolated in this work. From left to right: 1. DNA ladder, 2. Duck pond, 3. Kate 2, 4. Lacey, 5. 4NSE, 6. Rock Canyon, 7. Lehi Park, 8. Wayne, 9. RC2 10. Temple, 11. GMA house

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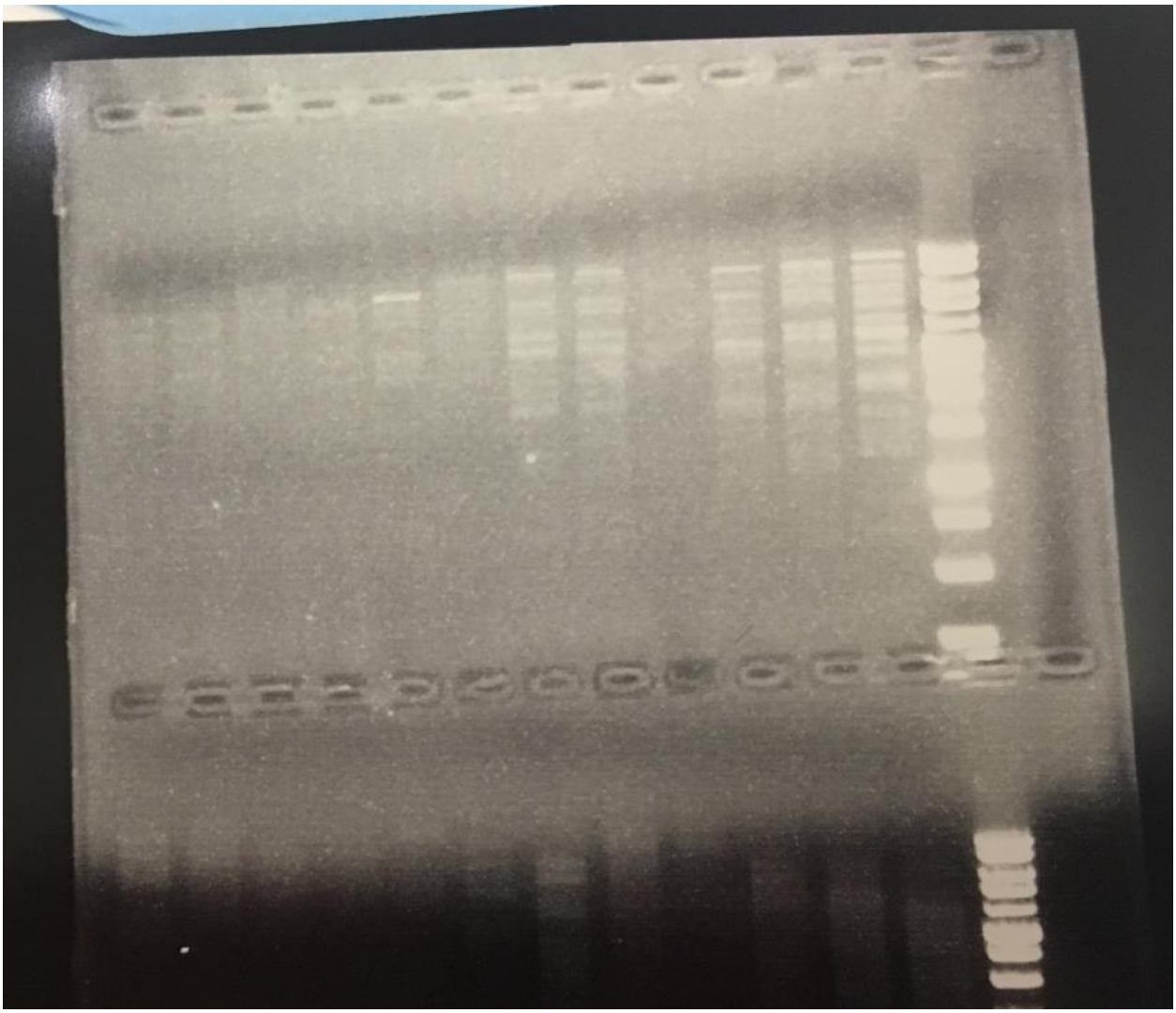

Figure 9. A gel showing the *Hind*III digestions of various phage genomes isolated in this work. From left to right: 1. R house, 2. Milo, 3. Timpview, 4. Mom's Garden, 5. Backyard GMA, 6. Gutter 7. Ryans Corner, 8. Harvey Fletcher, 9. FOB, 10. Elko Tree, 11. Nate 2, 12. Elko Garden, 13. DNA ladder. From the picture lanes 7 and 8 (Ryans corner and Harvey Fletcher) appear highly related.

Electron Microscopy

Electron microscopy was used to document the morphology of the phage involved in this study. Two images of different phage are shown in figures 10 and 11, illustrating phenotypically distinct phage capable of infecting *B. anthracis*.

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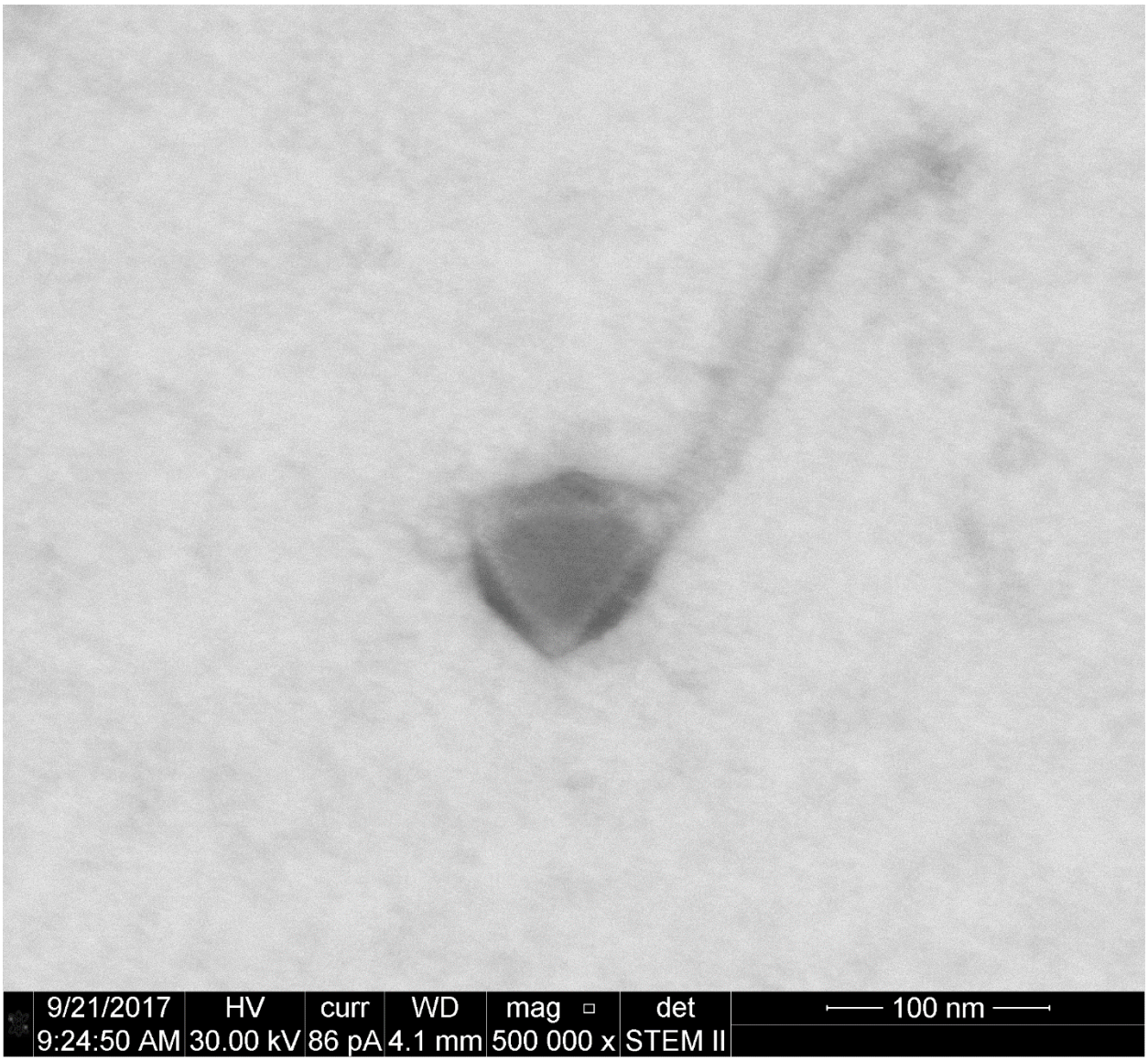

Figure 10. R House 2 phage sample analyzed by electron microscopy. A structure typical of the *Siphoviridae* is shown.

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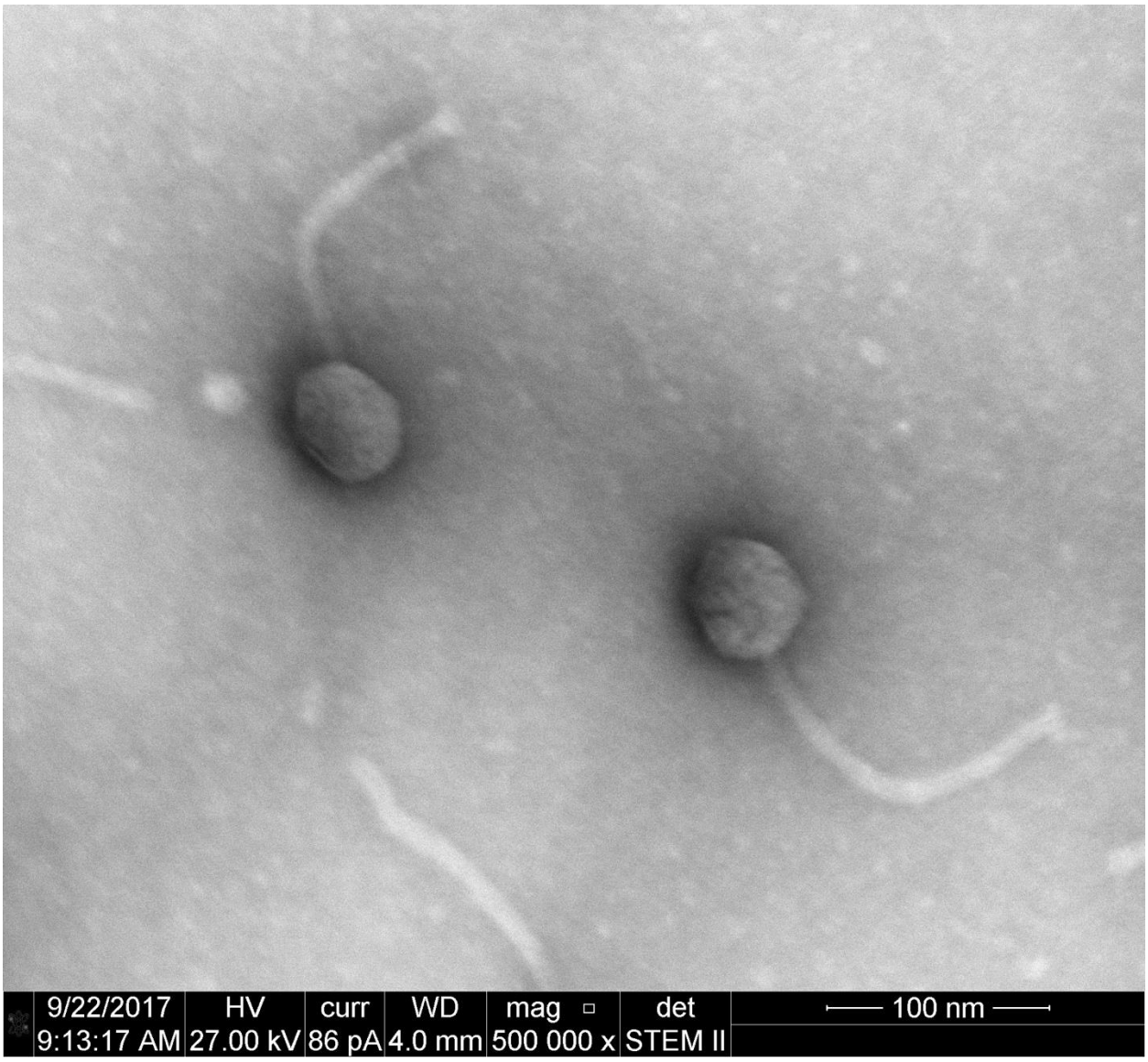

Figure 11. Backyard GMA phage viewed with electron microscopy at 500,000x magnification. Similar to figure 10, the structure of a *Siphoviridae* was observed.

Spot Tests

Spot tests were performed on Sterne and virulent *B. anthracis* strains. A summary of the spot test results is shown in Table 3. Most of the spot tests performed were done with 8 spots, two per phage, testing four phage against a single bacterial strain. Images of typical spot tests are shown in Figures 12, 13, 14, and 15.

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Table 3. Results from the spot test assays for 23 phage tested against 11 virulent *B. anthracis* isolates and the Sterne strain. Negative for infection (shown in Red), positive for infection (no color), and weak positive (depicted in yellow). Xs are shown for assays that were repeated at least 12 or more times.

		Bacteria Strain											
		34	39	102	158	193	293	402	442	462	488	489	sterne
	Phage #	34	39	102	158	193	293	402	442	462	488	489	
R House 2	1	positive	X positive	positive	positive	X positive	positive	X positive	positive	X positive	positive	positive	positive
RC2	2	X positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
MILO	3	positive	positive	positive	positive	positive	positive	X postitive	positive	X positive	positive	positive	positive
Timpview	4	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
GMA House	5	positive	X positive	X positive	positive W	positive	positive	positive	positive	positive	positive	positive	positive
Moms Garden	6	negative	positive	X positive	positive	positive	X positive	X positive	positive	X positive	X positive	X positive	positive
Lehi Park	7	X positive	X positive	X positive	positive	X positive	X positive	X postive	X positive	X positive	X positive	X positive	positive
Backyard Gma	8	negative	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
Gutter	9	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
Kate2	10	negative	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
Duck POND	11	X negative	X positive	X positive	X positive	X positive	X positive	X positive	X positive	X positive	Xpositive	Xpositive	positive
Ryans Corner	12	positive	positive	X positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
Harvey Fletcher	13	positive	positive	positive	positive	positive	positive	positive	positive	X positive	positive	positive	positive
Rock Canyon	14	negative	positive	positive	positive	X positive	positive	positive	positive	positive	positive	positive	positive
FOB	15	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
Temple	16	negative	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive	positive
Wayne	17	positive	X positive	X positive	positive	X positive	X positive	X weak positive	positive	X positive	positive	Xpositive	positive
Elko tree	18	positive	X positive	positive	positive	positive	X positive	positive	positive	X positive	Xpositive	Xpositive	positive
Nate2	19	positive	X positive	X positive	X positive	X positive	X positive	X positive	positive	X weak positive	X weak positive	Xpositive	positive
4NSE	20	positive	X positive	X positive	X positive	positive	positive	X weak positive	positive	X positive	Xpositive	Xpositive	positive
Lacey	21	positive	X positive	X positive	positive	X positive	X positive	X positive	positive	X positive	positive	Xpositive	positive
Elko Garden	22	positive	X positive	X positive	X positive	X positive	X positive	X positive	positive	X positive	Xpositive	Xpositive	positive
MARB	23	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative	positive

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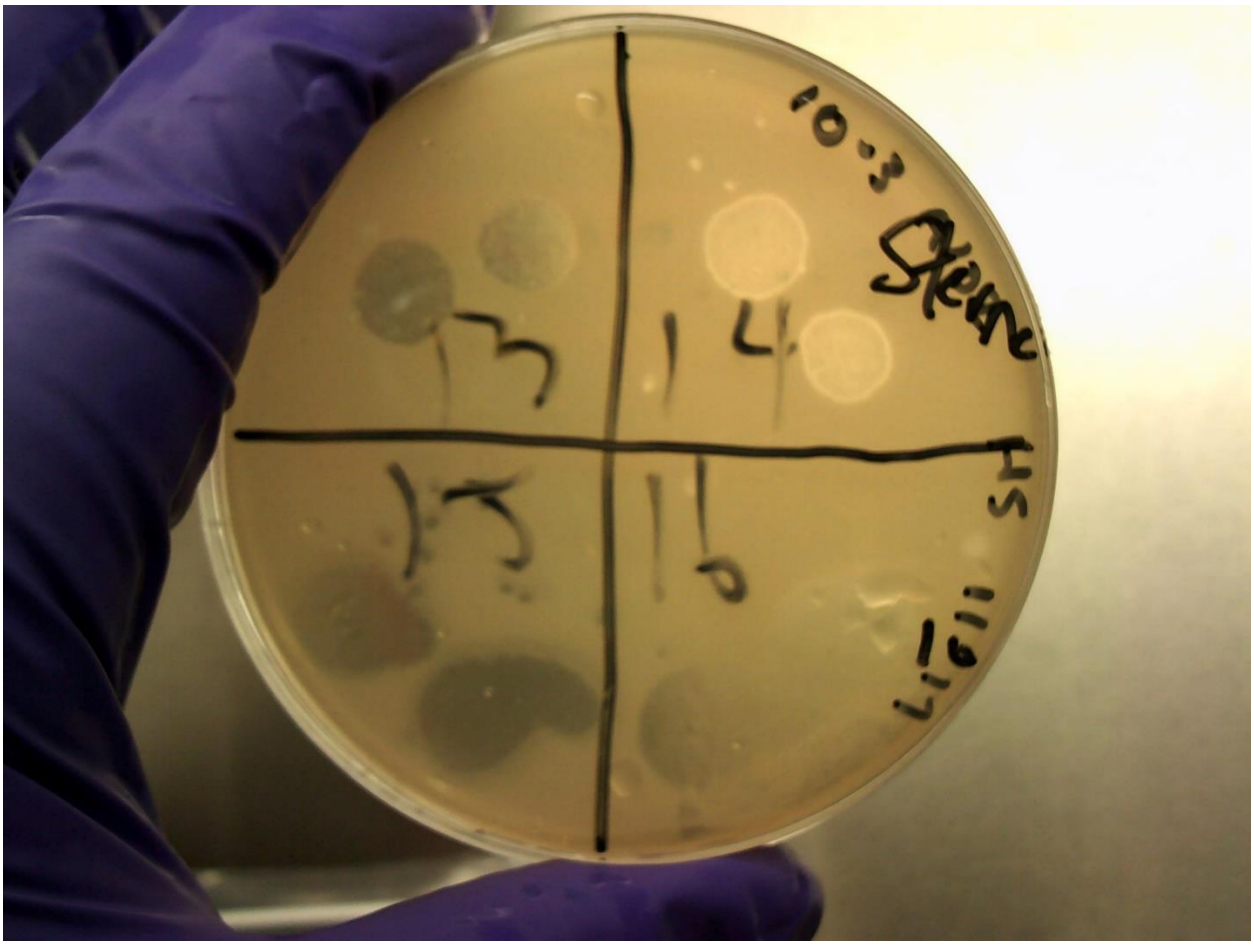

Figure 12. A typical spot test with eight drops, two drops per quadrant. This is a positive control plate showing that this phage infects the Sterne strain of *B. anthracis* with phage 13 (Harvey Fletcher), 14 (Rock Canyon), 15 (FOB), 16 (Temple). Varying levels of infection are evident.

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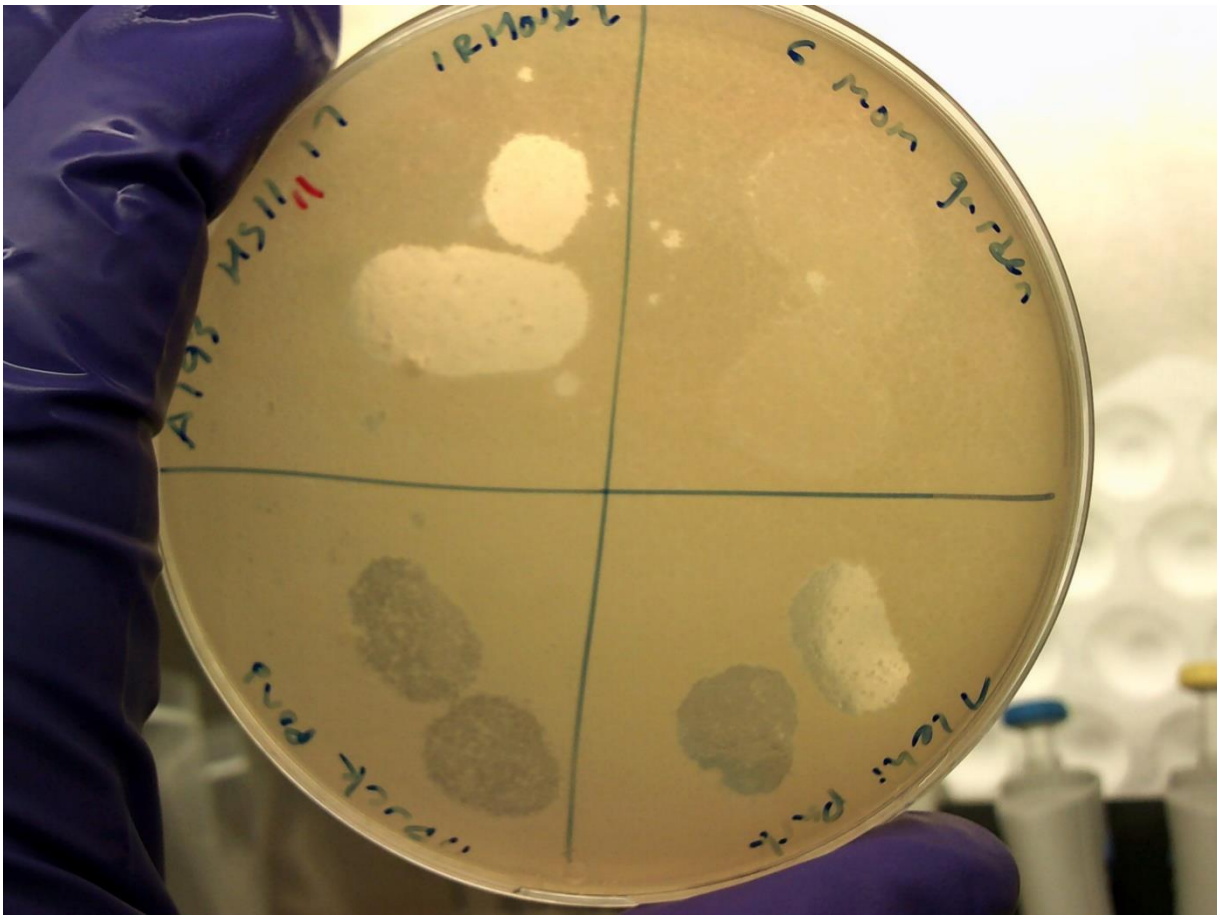

Figure 13. A typical spot test using the virulent strain of *B. anthracis* A193 with phage 1 (R House 2), 6 (Mom's Garden), 7 (Lehi Park), and 11 (Duck Pond). Similar to Figure 12, different phage expressed different infection characteristics.

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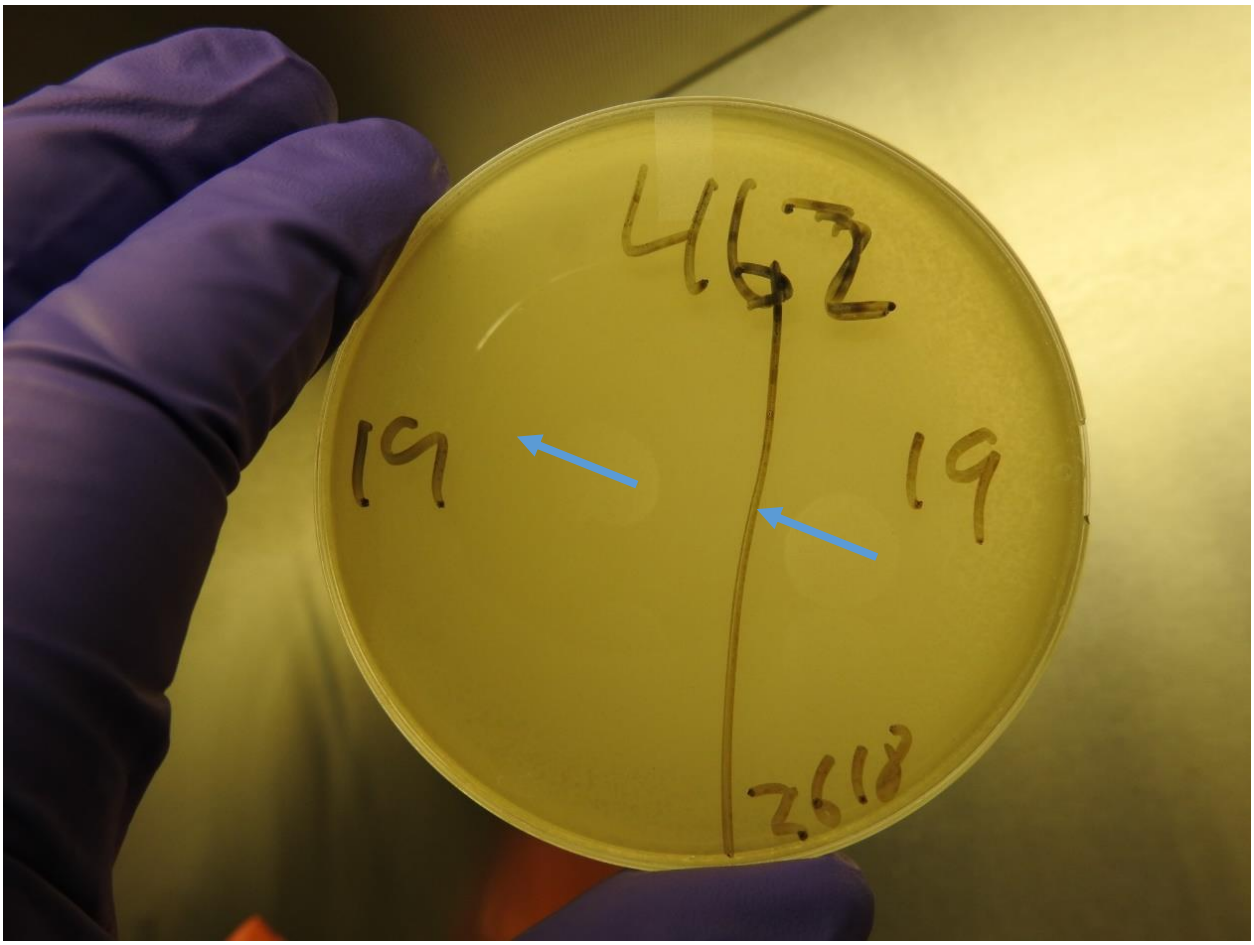

Figure 14. A spot test representing a weak positive infection of virulent *B. anthracis* strain A462 by phage 19.

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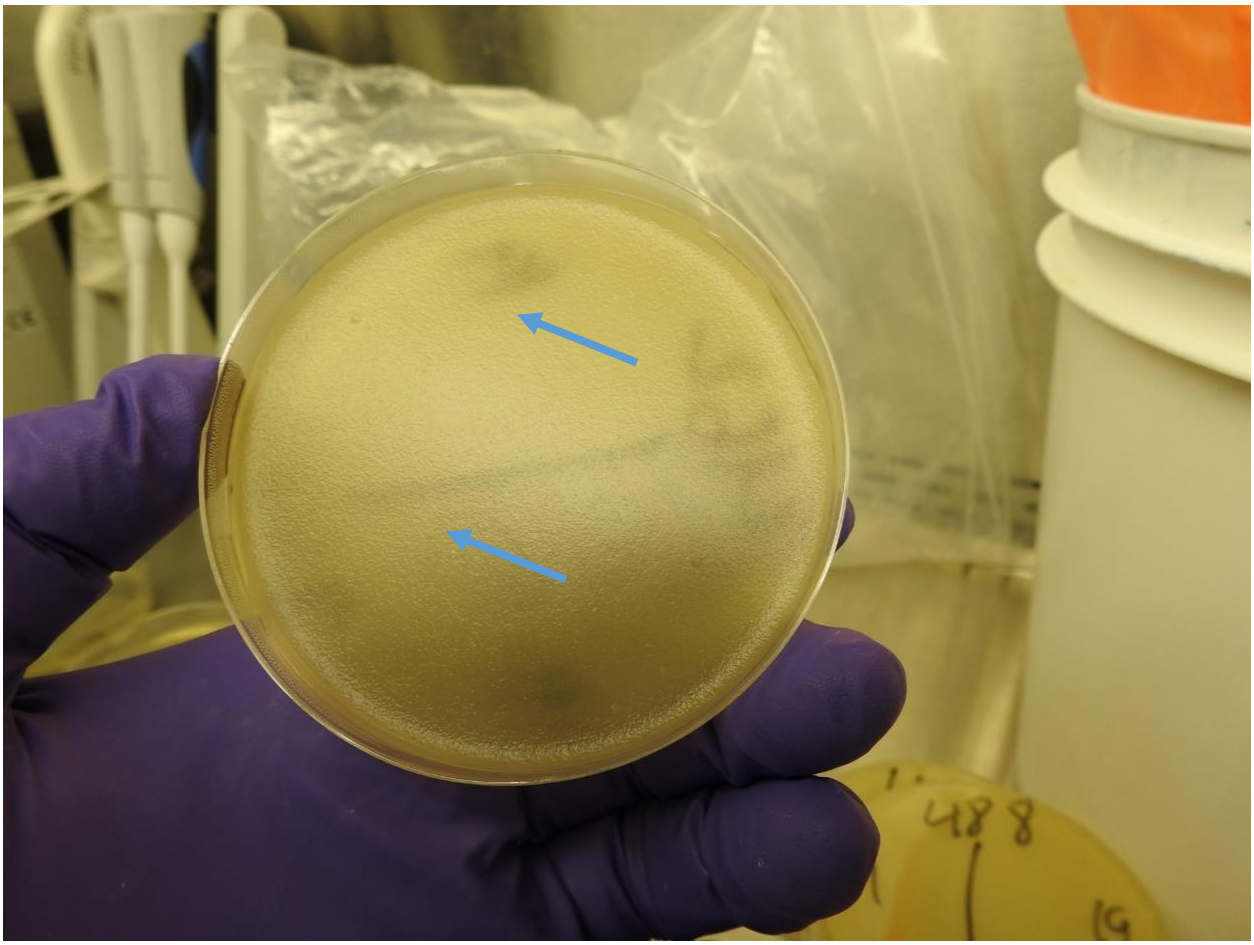

Figure 15. Same spot test and petri plate as Figure 14, but observed from the other side of the plate, showing how some weak positive results are difficult to see from both sides of the agar.

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DISCUSSION

Purpose

The purpose of this study was to identify phage that could infect virulent strains of *B. anthracis* and to date, only a handful of phage have been described that are capable of infecting this pathogen. (Alkalay et al., n.d.; Gillis & Mahillon, 2014; Minakhin et al., 2005; Sozhamannan et al., 2008). With a renewed interest in phage therapy, such phage could prove to be clinically useful. *B. anthracis* is easily weaponized, and has been used in bioterrorism attacks (Jończyk-Matysiak, Kłak, Weber-Dąbrowska, Borysowski, & Górski, 2014; H. Kikkawa, Fujinami, Suzuki, & Yasuda, 2007; H. S. Kikkawa, Ueda, Suzuki, & Yasuda, 2008; Sternbach, 2003). Preventive measures, such as large scale antibiotic prophylaxis can be expensive, as was seen in the 2001 anthrax attacks in the U.S. Phage are useful in fighting bacterial infections, as they are non-toxic to the larger environment, specific to their targets, replicable, inexpensive, and promising, as research is only starting to come forward after being abandoned for decades in favor of antibiotics (Abedon, 2015; Brüssow, 2012; Gill et al., 2015; Torres-Barceló & Hochberg, 2016a; Wittebole et al., 2013). This work characterized phage found to infect the non-pathogenic *B. anthracis* Sterne strain using enrichment and plaque purification techniques. It further discriminated among the phage found using genetic and phenotypic differences by employing restriction digests and electron microscopy, and finally tested phage for infectivity against a variety of virulent *B. anthracis* isolates in a BSL-3 laboratory using spot assays.

Phage Discovery

Phage discovery was completed in a BSL-2 lab before phage were tested in a more regulated and technically challenging BSL-3 facility (Wurtz et al., 2014). Figure 5 depicts a positive infection of *B. anthracis* Sterne using an enrichment culture from sample 17, Wayne.

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This image shows how phage discovery can successfully identify phage with a desired specificity. Simple techniques are used to find phage and phage discovery can be a new and inexpensive pathway to help fight antibiotic-resistant strains of bacteria (Allen et al., 2014; Rahmani, Zarrini, Sheikhzadeh, & Aghamohammadzadeh, 2015; Torres-Barceló & Hochberg, 2016b).

There are an abundance of phage around us and they have a remarkable capacity to control bacterial growth in many different environments (Abedon, 2009; H.-W. Ackermann, 1998; H. W. Ackermann, 2003). It is important to note that the concentration of phage in the enrichment culture was likely low, as only a small and easily countable number of plaques were produced on the plate. Therefore, enrichment cultures are unlikely to have a high enough titer of virus to be truly efficacious for any clinical work. However, high titer lysates can be easily and quickly prepared, as shown in Figure 7. The ability to prepare high titer lysates is absolutely essential to achieving a therapeutic benefit in phage therapy (Sarker et al., 2012).

Comparing Figures 5 and 7 shows a clear quantitative difference between an enrichment culture and a high titer lysate. The dramatic increase in PFUs is only possible by creating high titer lysates. High titer lysates are better able to achieve therapeutic success, as they contain a large enough number of phage to infect and destroy the target hosts (Ryan, Gorman, Donnelly, & Gilmore, 2011). A high phage concentration is one of the most important aspects of any successful phage therapy. If a phage is identified and it is impossible to amplify it to efficacious levels, it will likely prove worthless in a clinical setting. The phage investigated in this study could all be grown to high titers. Figure 7 shows the plates of a Wayne phage sample from a high-titer lysate, plated undiluted, and diluted 10^-1, 10^-3, and 10^-5. Phage titers varied among the phage, again showing that even with a monophyletic strain such as *B. anthracis,* the

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phage that infect it can be diverse and distinct. Figure 7 clearly shows that the HTL of Wayne lyses the bacteria so completely that no individual plaques can be seen in the undiluted sample. However, *B. anthracis* is able to form spores which seem more refractile to phage lysis (Walther, 2003).

It appears from the image that at a lower concentration of phage, more colonies from what we believe to be germinated spores, are seen on the 10^-1 plate when compared to the undiluted plate. It is intriguing that these colonies from spores are not as clearly seen on the 10^-3 and 10^-5 plates. Perhaps better infectivity occurs at a lower concentration of phage. In any case, little bacterial growth is seen on any of the plates until the 10^-5 dilution, where multiple individual plaques on a lawn can be seen. The reason why bacterial colonies do not appear on the 10^-3 and 10^-5 plates may be because in these plates, there was greater amplification possible for the phage. The higher relative concentration in the HTL plates may have killed all the bacteria quickly enough that only spores were left, hence the higher concentration of colonies on the undiluted plate. For the 10^-1 plate it would make sense that the colony count would be lower, as greater amplification of phage was possible because the phage did not instantly kill all the vegetative bacteria available.

Phage Discrimination

Phage discrimination is essential in phage biology. It is estimated that there are at least 10 different phage for every strain of bacteria (Rohwer, 2003). Phage are the most abundant biological entity on the planet, and it is estimated that there are more than 10^31 phage causing 10^23 infections every second (Hendrix, 2003). With so many environmental phage for each bacterial strain, it would be easy, albeit expensive, to sequence multiple phage. The concern is that it is possible to over sequence and have enough sequence similarity to question the scientific

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significance of the data if the environmental phage are closely enough related. Phylogenetic studies using EM data and genetic work done using restriction enzyme genome digests can better help scientists make decisions on which phage to study in depth, using sequencing, annotation, and other methods, without doing work that is redundant. Of the twenty-three phage used in this study, only one did not produce a digest pattern: MARB sample 23. This was also the only phage that was not able to infect the virulent strains. There are issues with this MARB phage that still need to be worked out. This phage was harder to manipulate than the other phage in this study. The only phage from the digestions that looked quite similar were Ryan's Corner and Harvey Fletcher (Figure 9, wells 7 and 8). These data seem to indicate that the other 21 phage are different from each other and could be sequenced. Sequencing and gene annotations are currently being performed on the phage in this study.

Electron microscopy is an essential technique in phage work and historically helped prove that phage were in fact viruses (H. W. Ackermann, 2003). Phage can vary greatly in their size and shape, but the phage we saw most often in our EM work belong to the *Siphoviridae* family (long, non-contractile tails). These are the most common phage in existence, making up over 60% of observed phage (Petrovski, Dyson, Seviour, & Tillett, 2012). We can see in Figures 10 and 11 that the viruses are most likely *Siphoviridae* from the tail and the head size and shape. At the same magnification, it appears that R House 2 is a larger phage than Backyard GMA.

Spot Testing

Spot tests are a common way to determine host range and infection potential and do so in a manner much quicker than plaque assays. Many recent papers have utilized spot tests to show infectivity of phage on their respective hosts, with hosts that ranged from Methicillin resistant *S. aureus* to *Esherichia coli* to *Klebsiella pneumoniae,* treating skin infections, foodbourne illness,

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and urinary tract infections, respectively (Askora, Merwad, Gharieb, & Maysa, 2015; Rahimzadeh, Gill, & Rezai, 2017; Snyder et al., 2016; Sybesma et al., 2016). Despite prolific use of spot testing in phage work and investigators insistence that spot tests are statistically similar to plaque assays, it appears that spot tests likely overestimate host range and infectivity (Champagne & Gardner, 1995; Mirzaei & Nilsson, 2015). Despite these significant concerns, our investigations have presented the spot test data with full understanding and acceptance that further work is necessary before infectivity and host ranges can be determined with full confidence.

During the spot testing for these investigations, thousands of plates were spotted and analyzed. Despite the need for plaque assay work in the future, the spot testing performed was repeated a dozen times or more for most strains and phage and was generally consistent. Only a few representative photos from hundreds were presented so that the processes used could be comprehended. Figure 12 shows how four phage infected the positive control strain of *B. anthracis Sterne*. From the picture, it is clear that the phage spots differed in turbidity. It appeared that Rock Canyon did not infect as well as Harvey Fletcher, when looking at the spot test turbidity. In quadrants 15 and 16, the phage FOB and Temple appear to have produced greater lysis, with a zone of clearing that showed less bacterial growth inside the zone. Figure 13 showed clearly that despite the phage all being isolated from the *Sterne* strain before being applied to a virulent strain of *B. anthracis,* all of the phage caused varying degrees of lysis.

From our analysis of spots on the Sterne strain, it became evident that rather than characterize infection by a magnitude of infection such as ‘strong or weak’, a binary ‘infection or non-infection’ was far more useful. A few outliers were noticed that truly showed consistent weak infection. However, the main point of our investigations was simply to show infection vs.

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non-infection, regardless of the degree of phage lysis in the spot tests. Table 3 diagrams which phage where able to infect which strain of virulent *B. anthracis*. The outliers which were assigned the label of a "weak" infection were phage 17 (Wayne) infecting strain A402 and phage 19 (Nate 2) infecting virulent strains A462 and A488. Greater elucidation of phage host range will be much more easily ascertained when rigorous plaque assays are performed at the same stringent replication expectations that characterized the spot assay work in this investigation.

Positive infections were much easier to see compared to the few "weak positive" infections. This may not be clear when viewing just one side of the plate, but was quite evident when viewing all the images, especially Figure 15. Figure 13 shows four positive infections with R House 2 (1), Mom's Garden (6), Duck Pond (11), and Lehi Park (7). R. House 2 had the strongest infection followed by Lehi Park then Duck Pond, and finally Mom's Garden. Figure 14 shows a weak infection of strain A462 with phage 19. The image shows lysis that is difficult to differentiate from the weakest infection (Mom's Garden) shown in Figure 13. However, when viewed from the front side in Figure 15, the infection of phage 19 into A462 is clearly not the same magnitude as Mom's Garden. Further testing will be needed to confirm or deny a presumptive infection. Only infections that were consistent and replicable were counted. This work, although important, is only the first part of a greater work which will substantiate the preliminary findings derived from the spot tests. Despite, the drawbacks of this study, it is still very important to identify phage that appear to show strong infection of virulent *B. anthracis.*

Summary and Future Directions

Twenty-three phage were found which were able to infect the Sterne strain of *B. anthracis*. Genetic and morphological characterizations were performed through restriction digests and electron microscopy, respectfully. Of these 23 phage, 22 showed activity against

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virulent strains according to our spot test data. While these findings set a strong basis for future work, there is additional work that needs to be completed, namely, plaque assays, genome sequencing, and inclusion of one *B. anthracis* strain that represents the "C" branch.

Plaque assays are much more definitive in their results and show substantial support that there is true infection occurring (Hyman & Abedon, 2010 Mirzaei & Nilsson, 2015). Bacteriocins and lysins present in the lysates used with spot tests can create 'false positives', as can "lysis from without" or simply a high concentration of phage that is logarithmically higher than the number of bacteria (Abedon, 2011). Plaque assays will be an important follow up to our present work.

Genetic sequencing of these phage is already underway and annotations of the phage genomes will be provided in the near future. The host range data, EM work, and restriction digest data are only a partial description of the phage listed in this study. Finally, a small but easily remedied issue with our study is that a representative of the C branch was not included in our host range spot test assays. The difficult to acquire and newly discovered C branch is represented by only one strain of the 221 strains of unique *B. anthracis* isolates in the study of the phylogeny of *B. anthracis* (Van Ert et al., 2007). Only four C branch isolates are known, and they do not express the marked pathogenic potential characteristic of the A strains. It is believed that B (15% of known strains) and C strains (less than 0.5% of known strains) play a more regional role in their environments, while A strains of *B. anthracis* are truly global in their scope and impact. It is not clear if A strains possess greater fitness or if their worldwide distribution is due to more stochastic processes. (Pearson et al., 2004). While missing a representative the C branch strains in our analysis, as they are new to the research scene and not common in any

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anthrax outbreaks, this discrepancy is somewhat mitigated by their lack of historic importance in bioterrorism research (Derzelle, Aguilar-Bultet, & Frey, 2016; Paul Keim et al., 2004).

Phage work is an exciting frontier with incredible growth potential in therapeutics, novel phage discovery, and uncovering keys to better understand unknown underpinnings in molecular biology. Although phage are the most abundant biological entity on the planet, much work is needed to better elucidate phage/host interactions and how to bring phage therapy into the 21st century (Petrovski et al., 2012). While our project identifying phage that can infect virulent *B. anthracis* is far from complete, this work provides the structure to start new efforts that will expand our knowledge of phage and *B. anthracis* alike, and provide additional opportunities to showcase the diversity and importance of the microbial world.

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