Discovery of a novel Bacteriophage capable of combating pathogens within the Bacillus *cereus* species group

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Introduction

The Bacteriophage- the smallest and most abundant being on Earth- has the potential to decelerate a rapidly progressing crisis known as the “post-antibiotic era”. With a name that literally means “bacteria eater,” bacteriophages (*phages* for short) are a form of virus that can be found nearly everywhere bacteria is found, ranging from the cold depths of the oceans to the insides of the human body. Essentially acting as natural nanomachines, the bacteriophages’ capability to destroy bacteria has been fine-tuned for millions of years, and current research makes it clear that phages are significantly more efficient than man-made antibiotics (Ling et al., 2022). While bacteria are more than capable of evolving to overcome antibiotics, phages are an entirely different battle.

Bacteriophage therapy is not a new concept, but the interest in its research went completely dormant after the invention of antibiotics following World War II. Not only were millions of lives saved due to antibiotics, but many significant medical breakthroughs were finally made possible, including organ transplantation and chemotherapy (Ling et al., 2022). For these reasons, antibiotics are rightfully considered to be one of the most effective breakthroughs in the history of medicine. However, bacteria have now had nearly 100 years to evolve slyly and rapidly, and many have grown resistant to antibiotics. Approximately 700,000 people die from antimicrobial resistance yearly, and this number is continuing to grow each year (Ling et al., 2022).

The post-antibiotic era could be disastrous without an alternative, leading researchers straight back to phage therapy.

Each individual phage has its own specific preferences for which form(s) of bacteria it will infect, known as a “host range”. A phage can have a narrow host range, a broad host range, or even a polyvalent range, rendering it capable of infecting more than one species of bacteria.

Potentially thousands of phages can be found within small samples of soil or water. If there’s a certain type of bacteria that researchers want to find a treatment for, this can be done by adding specific strains of bacteria to a sample containing phage. If the phage sample causes any significant destruction to the bacterial lawn, its effects will be visible after incubation. These visualizations are known as *plaques* and appear as clear spots that show exactly where phages have eradicated any present bacterial cells. Because viruses lay completely dormant unless they’ve detected a host cell, any nearby phages that don’t have this specific bacterium within their host range will remain inactive. The ones that do affect the bacteria will be the cause of the plaques, and they can easily be isolated and purified for further research.

Phage research has also been able to provide new information about the bacteria that phages infect, mainly regarding how they behave as hosts. One example of this is a newly discovered phenomenon, *Transposon mutagenesis*, which is a technique that bacteria can use to develop resistance to bacteriophages (Carson et al., 2018). While examining viral replication in bacterial hosts, bacterial cells can display the ability to trigger genetic mutations on their own receptors (specifically the receptors where bacteriophages bind & lyse). These receptors are not yet thoroughly understood.

By characterizing more novel bacteriophages and studying the interactions between the phages & bacteria, this research may lead towards an overall deeper understanding of bacterial characteristics, genetics, and behaviors. Going forward, bacterial behavior will be important to closely monitor, so as to not re-create another post-antibiotic era for future generations.

A few species of Bacillus currently have the attention of many phage researchers. Within the Bacillus genus, six notable and closely-related species make up the "Bacillus *cereus* species group". Out of those six, three are known to be pathogenic: one is responsible for anthrax (B. *anthracis)*, another commonly causes food poisoning (B. *cereus)*, and another is the world's most common biopesticide (B. *thuringiensis*). These three species pose infectious threats to humans, livestock, and insects, and can all act as opportunistic pathogens that cause tissue necrosis, food poisoning, and pulmonary infection in humans and animals (Kolstø et al., 2009). Although they differ in their virulence factors, they're so closely related genetically that many scientists believe that these six species should be considered as one singular species (Ehling-Schulz et al., 2004). B. *cereus* and B. *thuringensis* are so analogous that if B. *thuringensis* loses its plasmid, it then loses its three unique toxins (cry, cyt, and vip). These toxins are the only genetic trait setting it apart from B. *cereus*, rendering them indistinguishable from one another (Nikolaidis et al., 2022). While most cases of food poisoning point to B. *cereus* as the culprit, an unknown percentage of those cases may actually be caused by B. *thuringensis*.

So far, 38 novel bacteriophages for *B. cereus* have been discovered and analyzed (Kazantseva et al., 2022). Since the six species within the B. *cereus* species

group are so genetically similar, theoretically, bacteriophages for *B. cereus* may be able to successfully lyse B. *anthracis* and B. *thuringiensis,* which would effectively provide legitimate phage therapy for all three pathogens. Genomic research has already shown that out of the four prophages integrated into the B. *anthracis* chromosome, two of them have been found in other B. *cereus* group strains (Kolstø et al., 2009).

Similar to the “clustering” of the B. *cereus* species group, there is an effort being made to figure out a precise clustering system for phages as well, one example being considered Mycobacteriophages (Sauder et al., 2016). This study focused on figuring out a more-efficient classification system for bacteriophages. By examining the lysing behavior of 8 different novel bacteriophages that were found and studied by university students around the east coast, researchers found that while B. *cereus* phages typically have wide host ranges, their efficiency is nearly half as effective at lysing some familiar strains of bacilli.

The main objective for this research is simple: find, isolate, and characterize a B. *thuringiensis* phage (specifically B. *thuringiensis kurstaki*) to provide research data for future studies and experiments. In this study, a soil sample was collected at Mott’s Run Reservoir in Fredericksburg, Virginia. After isolation, potential phages found in the sample were enriched with BTK, causing only the phages that infect *Bacillus* bacteria to make visible plaques on the surface of the agar plates. The phages’ genome characteristics were identified. This study ultimately aims to sequence the genome of a novel bacteriophage to apply this phage to a broader range of *Bacillus cereus* bacteria.

Materials & Methods: Enrichment, Isolation, Plaque Assay, and Serial Dilution

A soil sample was collected from a muddy fishing peg located at Mott's Run Reservoir in Fredericksburg, Virginia around 9:00 AM, on 8/22/22. The specific coordinates for the fishing peg are 38.318647, -77.559669. The area in front of this peg, the *swim*, frequently contains a large number of fish, reeds, and vegetation. Due to the nature of the reservoir, the water level is constantly sinking and rising. This fishing peg often becomes flooded with water and its short wooden walls occasionally provide a home for tadpoles, tiny fish, insects, worms, and flora. The hope is that the local phages here are as interesting and dynamic as their biological counterparts.

To begin, potential phages were provided with a bacteria-rich environment. One gram of the sample was enriched with 3 mL *Bacillus thuringiensis* subspecies Kurstaki and 45 mL Trypticase soy broth (with 0.4% agar at 55°C) in a 250 mL baffled Erlenmeyer flask. This mixture was incubated at 30°C and agitated at 180 rpm for 24 hours. Contents were then transferred to a 40mL conical tube and centrifuged at 3000 rpm for 10 minutes. To filter out any bacteria, the liquid was poured into a new 40 mL conical tube and filtered through a 0.22µm filter unit and vacuum system. Samples were stored at 4°C.

One hundred µl Bacillus *thuringiensis kurstaki* was mixed with the phage sample and left undisturbed for 10 minutes. This mixture was then mixed with 3mL T-soy broth and plated on a T-soy agar plate. A second agar plate functioned as a control plate and was only plated with 100µl *B. thuringiensis* and 3mL T-soy broth. Plates were incubated for 24-48 hours at 30°C.

4 microcentrifuge tubes were filled with 100µl SM buffer (50 mM Tris, 8 mM $MgSo_4$, 100 mM NaCl, 0.01% gelatin, 1 mM $CaCl_2$, pH 7.5). With an inoculation needle, 4 plaques were selected and mixed into the SM buffer in each microcentrifuge tube. A new agar plate with 100 µl B. *thuringiensis kurstaki* and 3mL top agar was divided into quadrants. 3µl of each sample was added to each respective quadrant.

Samples from 2 of these plaques were then further isolated by mixing the contents into two new microcentrifuge tubes (pre-filled with 100µl SM buffer). Both samples were serial diluted to 10E-5. Only certain dilutions were plated, specifically 10E-4 and 10E-1, in hopes of producing one highly concentrated plate and one lowly concentrated plate. The plates were incubated for 19 hours at 30°C. The serial dilution process was repeated two more times, until a 10E-1 plate eventually produced a webbed plate.

Turning a webbed plate into a highly concentrated liquid phage sample

To generate a concentrated phage sample, the webbed plate was flooded with 5mL SM buffer atop the agar, and the buffer was allowed to penetrate the agar for 15 minutes. The contents were gently poured into a 50mL conical tube. The plate was rinsed with another 2mL of SM buffer to catch any remaining pieces, and this was also poured into the conical tube. The agar was agitated and broken, and the tube was centrifuged at 2500 rpm for 20 minutes. The lysate was filtered through a 0.22µm filter to catch lingering bacteria. A spot titer was performed with the newly filtered lysate. To degrade any remaining bacterial genetic material, 1mL lysate was mixed with 5µl nuclease. This mixture was incubated at room temperature for 30 minutes. The treated lysate was added to a new tube pre-filled with 2ml DNA clean-up resin. A Promega DNA clean-up kit was then utilized to isolate the phage's DNA. A column was attached to a syringe barrel, and 3mL of the resin/lysate solution was pipetted through the column, effectively only catching phage DNA. The column was washed with 2mL 80% isopropanol twice and was then centrifuged at 10,000*g for 5 minutes. Phage DNA was eluted twice with 50 µl ddH2O at 90°C.

To create agarose gel for electrophoresis, 0.8% agarose gel concentration was combined with 30 mL SM buffer (0.8g per 100ml, so ultimately 0.24g). The solution was heated and dissolved, then poured into a gel tray and allowed to solidify. The gel was

placed into the electrophoresis chamber and covered with SM buffer (about 1cm atop the gel). A ladder was loaded, as well as the phage DNA. Both were dyed for visibility. The chamber was covered, turned on, and adjusted to max settings at 90V. After one hour, the gel was imaged to display the DNA with a single band.

Results & Observations

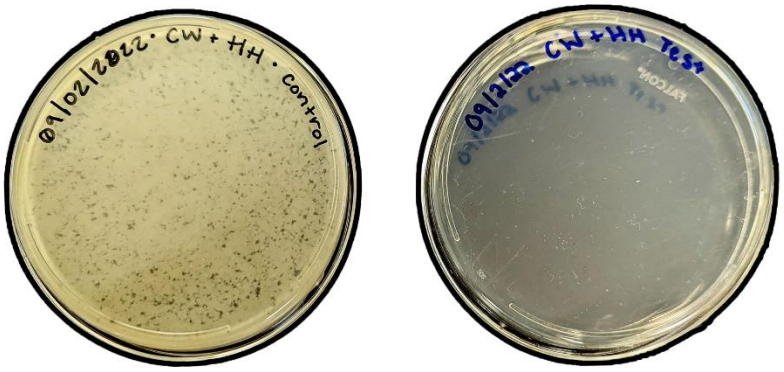

Figure 1: Results from two plates during enriched isolation.

The control plate (left) was only plated with100µl B. *thuringiensis* and 3mL T-soy broth, while the test plate (right) had the same contents plus the phage filtrate. The phage filtrate eradicated all of the bacteria present, leaving a perfectly clear plate behind.

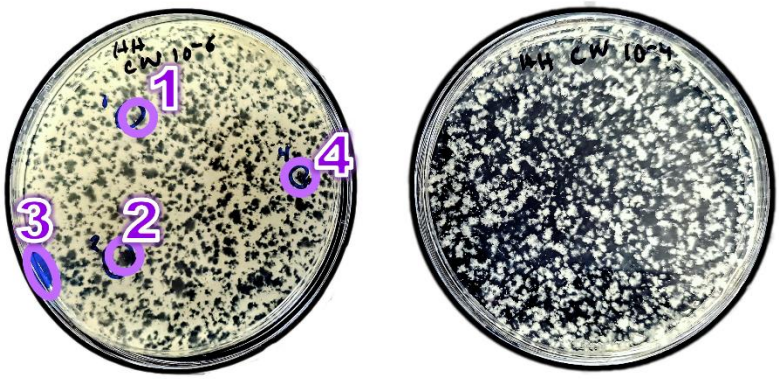

Figure 2: To impair phages, two more trials were done at dilutions 10E-6 plate (left) and 10E-4 (right).

After dilution, phages were sparse enough to generate distinct plaques. The 10E-6 plate (left) had small and blurry plaques, but they were otherwise sufficient for isolation. The plaques that were chosen to be further isolated (1-4) are nearly difficult to see here, but are circled & labelled in purple. The 10E-4 plate (right) ended up being too concentrated with phage to produce distinct plaques.

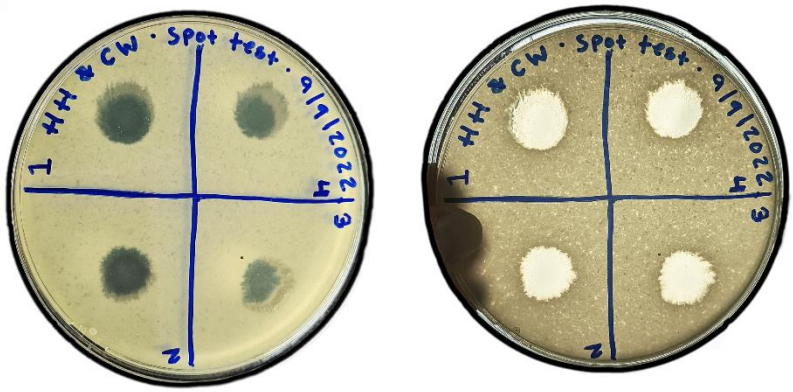

Figure 3: Plaque assay results from the four circled plaques on the previous plate (Fig. 2).

Samples from each plaque were topically added to their respective quadrant atop a bacterial lawn (with B. *thuringensis*), and each sample provided flawlessly clear plaques. All four samples would've been worthwhile to isolate.

*Both images are of the same plate; the image on the right shows the plate held up to a direct light source, for clarity

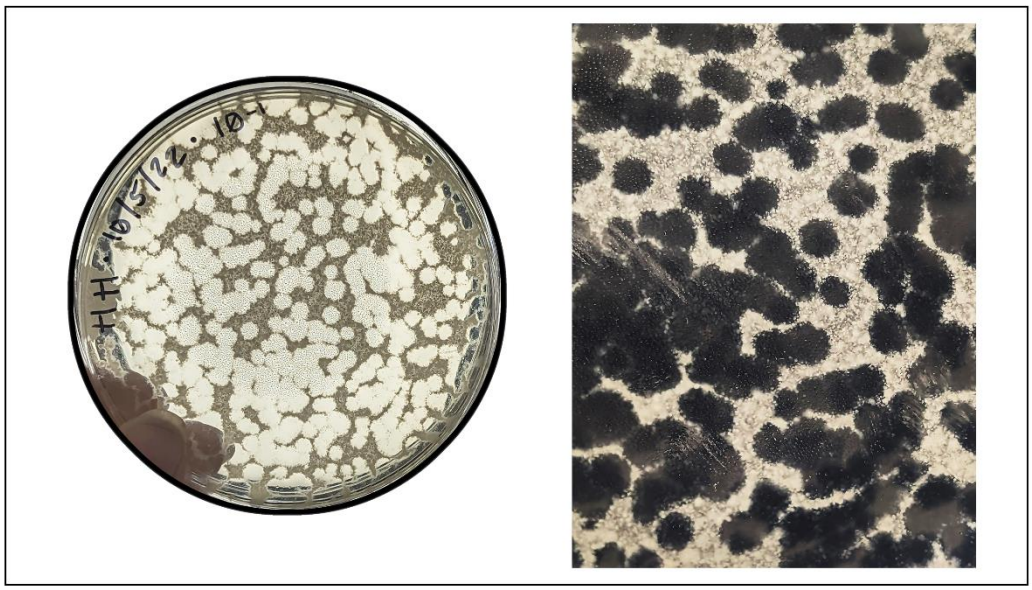

Figure 4: Webbed plate generated from plaque/spot 3 (from the previous plates in Figures 2 & 3), with a macro photo showing detailed plaque morphology.

Plaque/spot 3 was further isolated and purified until the phages finally produced this webbed plate. The undiluted sample was too powerful and generated a completely clear plate, but the sample was able to produce webbed plates at dilutions around 10E-1 (left). A macro photo showing details of the phage's plaque morphology (right) shows significantly more homogeneity compared to previous figures. Plaques are now all small and circular with fuzzy borders.

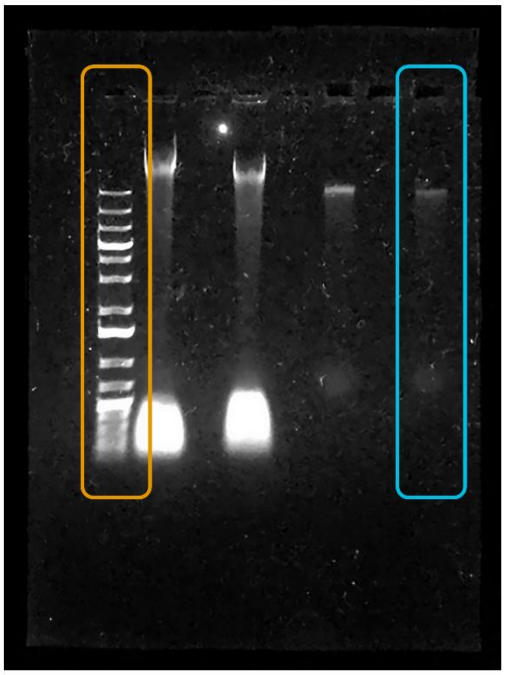

Figure 5: First run of gel electrophoresis results after the phage's DNA was isolated. The lane containing my phage's uncut DNA is within the blue box (towards the right), and the ladder is boxed in orange (towards the left). The three lanes in the middle of the figure are from lab partners.

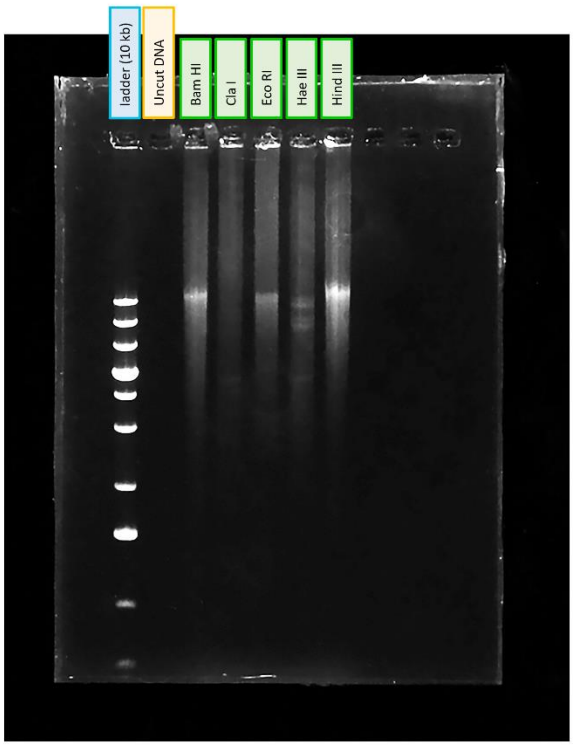

Figure 6: Restriction enzyme agarose gel (0.8%).

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