2-2018

Acinetobacter Bacteriophage Discovery in Soil

Michael Plaskett
Rose-Hulman Institute of Technology

INTRODUCTION

Acinetobacter Bacterium and Disease

The *Acinetobacter* genus is home to a wide range of bacteria that are gram-negative, aerobic, non-motile, oxidase-negative, catalase positive, and coccobacilli with a G+C content of 39 to 47% (Bergogne-Berezin, 1996). These bacteria are universal as they reside in the soil, water, sewage, various surfaces; and are also able to infect humans; however, a majority of the infections are caused by the species *Acinetobacter baumannii*. This bacterium is the most prevalent etiological cause of the *Acinetobacter* infections due to it being a nosocomial infection or one that is acquired in a health care setting (Bergogne-Berezin, 1996). The infections that occur in a healthcare environment are typically isolated to intensive care units and other settings with very ill patients (Bergogne-Berezin, 1996).

Challenges Associated with Treating *Acinetobacter*

Acinetobacter baumannii is capable of causing several diseases within an infected patient. These range from pneumonia to urinary tract infections. The symptoms presented vary depending on the specific disease (Bergogne-Berezin, 1996). Because *Acinetobacter baumannii* acts as a typical gram-negative, opportunistic pathogen, it infects areas such as the respiratory or urinary tracts. It is common for patients with exposure to mechanical ventilators to suffer from infections by *Acinetobacter baumannii* (Bergogne-Berezin, 1996).

These infections are difficult to treat as *Acinetobacter baumannii* exhibits multi-drug resistance and, as such, is hard to control (Maragakis, 2008). These drugs include penicillins, cephalosporins, and carbapenems. Polymyxins appear to be the most reliable drugs to use against *Acinetobacter baumannii*, but there are subtypes that exhibit resistance to this class of

drugs too (Maragakis, 2008). The high rates of antibiotic resistance in *Acinetobacter* species and specifically in *Acinetobacter baumannii* make it difficult to treat (Bergogne-Berezin, 1996). Next, the bacteria are able to survive for a long amount of time on surfaces and in many varying environmental conditions. Outbreaks of this bacterial species are common because it possesses these qualities (Maragakis, 2008).

Acinetobacter baumannii is not the only bacterial species that is difficult to treat; many other pathogens known by the acronym ESKAPE (*E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa*, and Enterobacter species) also have high rates of antibiotic resistance. These bacteria are pathogens that are commonly found in a healthcare setting and are resistant to a plethora of antibiotics (Hernandez, 2015). With each being handled on a case-by-case basis, a method to determine the subtype of the bacteria, to determine its resistance, would be very useful.

Phage Typing

We have evidence that bacteriophages and their use would be valuable to the medical for the identification and possible therapy of drug resistant and multi-drug resistant (MDR) bacterial strains. The healthcare community is seeing a rise in the number of MDR bacteria in primarily hospital settings. These bacteria cause many problems for the healthcare providers and the patients. MDR bacteria at the very least can extend hospital stays and thereby increasing all resources that go into that patient's visit and at worst can cause death (de Kraker, 2011). We believe that by using phages and phage techniques that these costs and deaths can be reduced. We hoped that through this work we would hope to contribute to the overall knowledge of phage and specifically to phage typing. Furthermore, we believe that with phage typing the problems listed can be alleviated. In phage typing, collections of bacteriophages are administered to the

bacteria isolate. The pattern in which the known phages lyse and kill the bacteria reveal the subtype of that bacteria (Bouvet, 1989). This information would be invaluable to the healthcare professionals, as it would allow them to choose antibiotics to which the bacteria are not resistant (Bouvet, 1989). Bouvet *et al*. have also proved this method successful for Acinetobacter in the late 1980s.

To accomplish phage typing, a set of known and characterized bacteriophages specific to that bacterial genus must be used. The lack of bacteriophages known to infect bacterial species is one hurdle that must be overcome to make the technique viable. A lack of up to date information categorized in databases presents another obstacle. (Baggesen, 2010).

Phage Therapy

Another phage technique that is being explored is phage therapy. In this technique, bacteriophages are administered to a patient with a bacterial infection with the purpose of the bacteriophages infecting and killing the harmful bacteria in the patient. As with all phages, they are specific to one species or subtype of a bacterial species (Baggesen, 2010). Due to this requirement, further research into phage therapy requires known characterized phage. Our project provides these known phages for both typing and therapy experimentation.

Currently, research is being done with phage therapy to prove its efficacy. Most interestingly, labs have demonstrated phage therapy's ability to improve the use of antibiotics. Through the administration of phage, targeted bacteria are presented with evolutionary tradeoffs that, in this study, resulted in loss of antibiotic resistance for *Pseudomonas aeruginosa* (Chan, 2016). Phage research is a promising field that requires large amounts of information and sets of standardized phage to which our project can contribute.

MATERIALS AND METHODS

Acinetobacter Culturing

Acinetobacter baylyi strains were obtained from previously existing cultures maintained by Rose-Hulman's lab manager Shannon Tieken. The existing cultures were t-streaked on MacConkey agar. A single bacterial culture was picked from that plate using a flame sterilized loop and t-streaked onto a Luria Bertani (LB) Agar plate. Furthermore, liquid cultures were created from the bacteria grown up on that LB plate. The liquid cultures were stored in test tubes containing 5 mL of LB broth. A single bacterial culture from the LB plate was removed using an autoclaved toothpick that was then placed into the LB broth and allowed to grow overnight at 37ºC and shaking at 280 RPM (Mattila, 2015).

Soil Collection & Isolation of Phage

Phage was isolated from soil samples collected on Rose-Hulman's campus. Soil was taken from an empty patch of ground located behind the White Chapel. Approximately, 1 gram of soil was collected and suspended in 6 mL of Luria-Bertani Broth. 300 µL of overnight grown *A. baylyi* culture was then added and the solution was incubated at 37ºC for 6 hours with shaking at 280 RPM. After the 6 hours, drops of chloroform were added to lyse bacterial cells possibly containing phage and the solution was left to sit for 15 minutes. Following that, the solution was subjected to vacuum filtration with 5.5cm 23 micron pore size Whatman Qualitative Filter Paper in order to remove soil and bacterial debris. The filtrate was then taken and spun at 11,000g for 5 minutes. PEG6000 and NaCl were then added to the supernatant at final concentrations of 10% and 1M respectively. The solution was stored overnight at 4ºC and spun at 11,000g for 20 minutes the next morning. The supernatant was discarded and the surviving pellet was dissolved in PBS. This dissolution was filtered with 0.2µm filters. The filtrate was taken and serially

diluted 1:10. Each dilution was taken and plated for plaques using a double plaque assay (Yang, 2010; Stenholm 2008; Carey-Smith, 2006).

Double Plaque Assays

250 mL of top agar was created in addition to regular LB agar and broth in order to perform the assay. Top agar was created from a 0.7% agar formulation of the LB agar recipe. Bacterial phage cultures were added to 2mL exponential phase *Acinetobacter baylyi* and incubated for 5 minutes. After incubation, the phage and bacteria were added to 6 mL ~45ºC melted top agar and poured onto a dried LB agar plate. The plates were allowed to cool and then checked after 4 hours for growth and 6 hours and overnight for plaque formation (Mocé-Llivina, 2004).

Purification of Phage

Single plaques were purified following isolation. To select one plaque a sterile Pasteur pipette was used and a plaque was punched from the agar and placed in SM buffer. The solution was then shook in 37ºC at 280 RPM for 2 hours. Chloroform was added to the solution at 50µL per 1 mL of SM buffer. Phage was separated into organic layer and added to exponential phase *A. baylyi* culture. This culture was then incubated for 20 minutes and used to re-perform a double plaque assay (Stenholm, 2008).

One-Step Growth Curve

The one-step growth curves used 0.7% LB top agar and 1.5% regular recipe agar. Exponential growth phase *Acinetobacter baylyi* was grown and incubated for two hours at 37ºC and 280RPM. 10 mL of the growth phase culture was infected at an MOI of 0.01 by a prepared culture of phage from the purification step. The solution was left to adsorb for a total of 5 minutes. After the 5 minutes, the solution was centrifuged in order to remove any excess phage.

The supernatant was discarded and the pellets were re-suspended in 5 mL of LB broth and incubated at 37ºC. Two sets of samples were collected every 5 minutes for 40 minutes. Each set was immediately 10-fold serially diluted and plated for titration. The second set was treated with chloroform to release intracellular phage. This set was then used to determine the eclipse period of the page. From the plaque forming units on the plates, the latent and burst size of the page were determined (Yang, 2010; Stenholm, 2008; Carey-Smith, 2006; Adams, 1959; Merabishvili, 2014).

RESULTS

Bacteriophage that infect *Acinetobacter baylyi* can be found in soil surrounding Rose-Hulman Institute of Technology. During Dr. O'Connor's microbiology class, students were able to isolate *Acinetobacter baylyi* from soil around Rose-Hulman's campus. Bacteriophages are found in areas where the bacteria they infect are found, because of those factors, we hypothesized that bacteriophages infecting *Acinetobacter baylyi* could be found on Rose-Hulman's campus. Soil samples were collected from unfertilized ground behind the White Chapel at the West end of campus. Following our isolation protocol, phage was acquired via verification and culturing with double plaque assays. These assays were used throughout the rest of the experiment for purification and amplification and were able to show that phage from *Acinetobacter baylyi* can be found at Rose-Hulman (Figure 1).

Following isolation, we were able to use that isolated phage existing in the assay for purification and amplification protocol. It is likely that the phage plaques present in the assays obtained from isolation contained more than one strain of bacteriophage. In order to obtain one specific strain it was necessary to perform successive rounds of plaque assays to pick and therefore isolate one plaque of bacteriophage. This purification was carried out using lab techniques delineated in the literature and three successive rounds of plating were achieved.

DISCUSSION

The ESKAPE pathogens identified as targets for MDR research have been studied with varying amounts of attention. Their threat to the future of patient care presents issues that should be studied along with alternatives to treatment (Hernandez, 2015). We think that alternatives lie in the use of bacteriophages for both phage typing and phage therapy. To adequately make use of those techniques large amounts of characterized phage must be understood and that information available. Our project focuses on those rudimentary steps necessary for the use of those advanced techniques.

Our project was successfully able to isolate bacteriophage from soil on Rose-Hulman's campus and purify a single plaque with successive rounds of double plaque assays. Due to a loss of those purified plates, characterization of the obtained plaques was not able to be accomplished in the given time frame of the experiment. In order to fully complete the project, then several experiments would need to be completed after reobtaining sets of purified, unknown phage. Each of these experiments involve characterization of a set of phages that would need to be obtained. The primary characterization technique employed would be restriction fragment length polymorphism (RFLP). RFLP would give us both the knowledge that our obtained set of phages are indeed different as well as a profile of those phages. Further DNA work would also be needed (Merabishvili, 2014). Upon verification of separate phages, DNA or RNA would need to be isolated and sent for sequencing to complete the main characterization of our phages.

Additional characterization experiments would also be needed, as phages are often classified on several different criteria. Plaque size and type are two different physical characteristics of the phages that are commonly used (Ackermann, 2010). Plaque size would be measured by simply taking the diameter of the acquired plaques, and plaque type of lytic or

lysogenic can be recorded through simple observation of the plaques themselves (Urban-Chmiel, 2015; Merabishvili, 2014). Lastly, activity of the phages would be characterized using a one-step growth curve. That assay would quantify the infective activity of the phages with their host bacterium (Yang, 2010; Stenholm, 2008; Carey-Smith, 2006; Adams, 1959; Merabishvili, 2014).

In the future, phages may have several applications ranging from typing to therapy. With a database of characterized phage made available, then phage typing methods could be used in the future in order to identify the subtypes of bacterial strains and help physicians to choose treatment options based on those phage profiles. Additionally, phage therapy has been gaining attention as a possible treatment option to use with antibiotic treatment. Phage therapy has the added benefit of a historical basis for its use dating back to World War I and extensive use in former Soviet Bloc states (Levine, 1992). Current research shows that phage therapy could be a viable treatment option to use with antibiotics to increase their effectiveness and further research may reveal that standalone phage treatments are a viable option for certain infections (Chan, 2016).

CONCLUSION

Our project focused on the discovery and identification of bacteriophage that infected *Acinetobacter baylyi*. We succeeded in isolating and purifying those phages from soil samples taken on the campus of Rose-Hulman Institute of Technology. Our project shows that phages infecting these bacteria may be found in the soil in which they inhabit and if their prevalence in the environment matches that *Acinetobacter baylyi*, then a great diversity of phage may be present. Phage research has the potential for many applications including phage typing and therapy. To make those applications viable, a great deal of collected and characterized phage need to be studied. This project aimed to contribute to that collection. It provides a base for future research at Rose-Hulman with the existing methods and strategies employed in this project.

BIBLIOGRAPHY

Ackermann, Hans W. "Bacteriophage Taxonomy." *Microbiology Australia* (2011): 90-94. Print.

Adams, Mark Hancock. *Bacteriophages*. New York: Interscience, 1959. Print.

Baggesen, D. L., E. M. Nielsen, and H. C. Wegener. "PHAGE TYPING OF SALMONELLA TYPHIMURIUM – IS IT STILL A USEFUL TOOL FOR SURVEILLANCE AND OUTBREAK INVESTIGATION?" *Eurosurveillance*. N.p., 28 Jan. 2010. Web. 10 Feb. 2016.

BERGOGNE-BE´RE´ZIN, E., and K. J. Towner. "Acinetobacter Spp. as Nosocomial Pathogens: Microbiological, Clinical, and Epidemiological Features." *Clinical Microbiology Reviews* 9.2 (1996): 148-65. Print.

Bouvet, P. J., S. Jeanjean, J. F. Vieu, and L. Dijkshoorn. "Species, Biotype, and Bacteriophage Type Determinations Compared with Cell Envelope Protein Profiles for Typing Acinetobacter Strains." *Journal of Clinical Microbiology*. U.S. National Library of Medicine, n.d. Web. 10 Feb. 2016.

Carey-Smith, Gwyneth V., Craig Billington, Angela J. Cornelius, J. Andrew Hudson, and Jack A. Heinemann. "Isolation and Characterization of Bacteriophages Infecting Salmonella Spp." *FEMS Microbiology Letters* 258.2 (2006): 182-86. Web.

Chan, Benjamin K., Mark Sistrom, John E. Wertz, Kaitlyn E. Kortright, Deepak Narayan, and Paul E. Turner. "Phage Selection Restores Antibiotic Sensitivity in MDR Pseudomonas Aeruginosa." *Nature News*. Nature Publishing Group, 26 May 2016. Web. 22 Feb. 2017.

Hernandez, Simon, Tiffany Tseng, and Jo Handelsman. "Our Mission." *The Small World Initiative: A Research Guide to Microbial and Chemical Diversity*. N.p., 2015. Web. 10 Feb. 2016.

Kraker, Marlieke E. A. De, Martin Wolkewitz, Peter G. Davey, and Hajo Grundmann. "Marlieke E. A. De Kraker." *Clinical Impact of Antimicrobial Resistance in European Hospitals: Excess Mortality and Length of Hospital Stay Related to Methicillin-Resistant Staphylococcus Aureus Bloodstream Infections*. N.p., 01 Apr. 2011. Web. 22 Feb. 2017.

Levine, Arnold J. *Viruses*. New York: Scientific American Library, 1992. Print.

Maragakis, Lisa L., and Trish M. Perl. "Antimicrobial Resistance: Acinetobacter Baumannii: Epidemiology, Antimicrobial Resistance, and Treatment Options." *Clinical Infectious Diseases CLIN INFECT DIS* 46.8 (2008): 1254-263. Web.

Mattila, Sari, Pilvi Ruotsalainen, and Matti Jalasvuori. "On-Demand Isolation of Bacteriophages Against Drug-Resistant Bacteria for Personalized Phage Therapy." *Front. Microbiol. Frontiers in Microbiology* 6 (2015): n. pag. Web.

Merabishvili, Maia, Dieter Vandenheuvel, Andrew M. Kropinski, Jan Mast, Daniel De Vos, Gilbert Verbeken, Jean-Paul Noben, Rob Lavigne, Mario Vaneechoutte, and Jean-Paul Pirnay. "Characterization of Newly Isolated Lytic Bacteriophages Active against Acinetobacter Baumannii." *PLoS ONE* 9.8 (2014): n. pag. Web.

Mocé-Llivina, Laura, and Francisco Lucena. "Double-Layer Plaque Assay for Quantification of Enteroviruses." *Applied and Environmental Microbiology*. N.p., 01 May 2004. Web. 22 Feb. 2017.

Pal, R. B. "Acinetobacter Calcoaceticus-an Opportunistic Pathogen." *Journal of Postgraduate Medicine*. N.p., 1981. Web. 22 Feb. 2017.

Stenholm, A. R., I. Dalsgaard, and M. Middelboe. "Isolation and Characterization of Bacteriophages Infecting the Fish Pathogen Flavobacterium Psychrophilum." *Applied and Environmental Microbiology* 74.13 (2008): 4070-078. Web.

Urban-Chmiel, Renata, Andrzej Wernicki, Diana Stęgierska, Marta Dec, Anna Dudzic, and Andrzej Puchalski. "Isolation and Characterization of Lytic Properties of Bacteriophages Specific for M. Haemolytica Strains." *PLOS ONE PLoS ONE* 10.10 (2015): n. pag. Web.

Yang, Hongjiang, Li Liang, Shuxiang Lin, and Shiru Jia. "Isolation and Characterization of a Virulent Bacteriophage AB1 of Acinetobacter Baumannii." *BMC Microbiology BMC Microbiol* 10.1 (2010): 131. Web.

FIGURES

Figure 1: Double plaque assay containing initial purification. Bacterial phage cultures were added to 2mL exponential phase *Acinetobacter baylyi* and incubated for 5 minutes. These phage were taken from soil from around Rose-Hulman's campus and isolated using vacuum filtration with 5.5cm 23 micron pore size Whatman Qualitative Filter Paper in order to remove soil and bacterial debris. That filtrate was spun into a pellet. These pellets were then dissolved in PBS and subjected to a 0.2 micron filter. This phage solution was then serially diluted and incubated. After incubation, the phage and bacteria were added to 6 mL ~45ºC melted top agar and poured onto a dried LB agar plate. The plates were allowed to cool and then checked after 4 hours for growth and 6 hours and overnight for plaque formation. Plaque formation indicates the presence of phage that infects *Acinetobacter baylyi* from the isolation protocol.