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Dr. Lewis
URES 197
Bacillus Bacteriophages
At the University of Mary Washington, the Biology 125 and 126 classes study bacteriophages in lab that each student has isolated. Unfortunately, the phage DNA does not store well for the long term. The original plan for this past semester was to recover phages from 2011 and design primers to run PCR for each phages sample. The phages were tricky though, taking a little over half of the semester to recover only nine phages out of 29: Megatron, Lukalei, Ninu, Rosie, Peanut, Tatou, Greenburg-6, Sakpata², and Storm-01. Since we were having so much trouble with them, and with the most recent class struggling to grow their phages attention was shifted to them and potential problems of Calcium in the buffers used was explored. All of the Trypticase soy agar (TSA) plates inoculated with Bacillus thuringiensis subspecies Kurstaki (B. thuringiensis Kurstaki) and different phages were kept since we have seen that it is more likely to recover phages from plates than DNA.

Almost all of the wet lab work involved plating 0.5 ml B. thuringiensis Kurstaki with 4.5 ml Top Agar and inoculating the bacterial lawn with phages. When working with the 2011 phage samples for the first time, three grid plates were made with each grid square getting a 10 µl drop of one of 29 different phages from the freezer. After incubating those plates for roughly 48 hours at 30°C, they were checked for plaques. Those samples that grew plaques then had 5 µl samples of DNA spotted onto 3, quartered plates to better isolate them and make sure that the perceived growth was not actually interference from another grid square’s phage. Two more spot plates with grids were made for the samples that did not show plaques and treated with 5 µl of DNA. The 13 that had grown directly from DNA sample spots were plated onto three new, divided plates only this time the plaques were picked with a micropipette tip from the first quartered plates instead of using DNA.
Unfortunately, there were no visible plaques on the new grid plates from the 16 samples that had not previously grown nor were there plaques from four samples from the plates with picked plaques. Those four samples had shown the weakest growth from DNA and did not infect when a plaque was used instead. All of those 20 samples were then enriched in 20 individual tubes containing 5 ml T-Soy Broth, 200 µl \textit{B. thuringiensis} Kurstaki, and 10 µl of respective phage sample. Those tubes were set on a shaker to incubate at 30°C before the shaker was turned off, but the samples remained in the incubator.

After incubation, the enriched tubes looked cloudy like they still had bacteria in them which did not bode well for plating. From each tube, one ml was taken and dispensed in a microcentrifuge tube which was spun and 10 µl of the different spun samples were plated on four divided plates. The remaining supernatant of about 850 µl – 875 µl was stored in new microcentrifuge tubes and the pellet-containing tubes were discarded. The plates were incubated at 30 °C overnight and yielded no plaques. It was then decided to leave those phages alone until we could figure out what to do with them.

In the meantime, the nine phages that had been recovered then had their host range tested. Bacterial lawns made with 4.5 ml Top Agar and 0.5 ml of \textit{Bacillus cereus} (\textit{B. cereus}), a close relative of \textit{B. thuringiensis} Kurstaki, were made on divided plates from plaques picked from \textit{B. thuringiensis} Kurstaki plates. Those were incubated at 30°C overnight. Nothing grew but we decided to resuspend the picked plaques in 20 µl SM Buffer to draw out the phages and spot plates of \textit{B. cereus} again. One of the phages, storm-01 was the only obviously lytic one and had a nice sized clear plaque in its grid. All eight other samples showed cloudy plaques, but plaques nonetheless. At this juncture is where I moved on to working with the 2013 phages since the
classes were having so much trouble and I was running out of time to design primers and accurately test them.

First plates dividing samples from Dr. Lewis and Dr. Loesser’s classes were made with each section of the bacterial lawn designated by the student initials. I had to pick plaques from plates from quite early in the semester. After incubation like all the other $B. \text{thuringiensis}$ Kurstaki plates from this semester, plaques had grown for everyone. The thought here was if there was a problem with the SM Buffer or the cultures of $B. \text{thuringiensis}$ Kurstaki.

$B. \text{thuringiensis}$ Kurstaki was ruled out because I personally had no trouble with it and I had been using it all semester as well, plus the earlier plates from the students were fine. That left the SM Buffer as the only possible culprit so I made some but scaled it down from a 1 L recipe to a 100 ml recipe. The 100 ml was then divided into 4 graduated cylinders with 25 ml each, one was left alone, one had only CaCl$_2$ added, one had only gelatin added, and the final one had both gelatin and CaCl$_2$ for which the original recipe calls. Along the way, it was discovered that the recipe calls for 10 ml of 100 mM CaCl$_2$ for 1 L and the calcium solution in the lab was actually 1 M. We wondered if that might not have been correctly diluted on accident, but we made sure to dilute it correctly for the 50 ml sample to which it would be added. These were stored in the microscopy preparation room for use later.

A few days after the SM Buffers had been made, each sample of SM Buffer had 1 ml filtered through a 0.22-micron filter with a syringe. After filtration, 20 µl of each type of SM Buffer was put into two new microcentrifuge tubes where a robust plaque and a weaker plaque were put into one microcentrifuge tube or the other. I chose Megatron for the robust growing phage and Lukalei for the weaker growing phage. The picked plaques were suspended for 20 minutes and then five µl from each microcentrifuge tube was plated on a $B. \text{thuringiensis}$
Kurstaki/Top Agar lawn and was distinctly labelled to know which type of SM Buffer in which the phages had been suspended. The plates were incubated over the course of five days at 30 °C. The filtered SM Buffers had been saved and 20 µl from each was dispensed into two new microcentrifuge tubes for all four kinds of buffer just like the week before. This time the plaques that were resuspended in the buffer samples were from the plates made with the new buffers. Both Megatron and Lukalei grew fine and did not show preference for any particular kind of buffer but the actual test is how they could handle it long term. Five µl of the suspended plaques were once again plated on a lawn of *B. thuringiensis* Kurstaki and Top Agar and left to incubate while the tubes with phage and SM Buffer were stored in the refrigerator for the course of 11 days.

Much like the time before, Megatron and Lukalei had grown well after soaking in any kind of SM Buffer for only 20 minutes. New plates of *B. thuringiensis* Kurstaki and Top Agar were made with five µl of the stored samples of Megatron and Lukalei in the buffers. Those were incubated at 30 °C overnight. There was an issue with the Top Agar that day so the plates were not made in as sterile of an environment as possible. The Top Agar/*B. thuringiensis* Kurstaki mixture had to be added right out of the hot water bath instead of taken next to the Bunsen burner and flaming the glass lip of the tube. Even so, it did not seem to affect the plates that much. The plaques did not grow well after being stored in any kind of SM Buffer but they showed even less growth or no growth in the buffers treated with CaCl₂. That concluded the semester, but there is more to look into next semester.

For the course of this past semester, the results have been obvious, such as whether plaques grew or not and they were quick due to the nature of *B. thuringiensis* Kurstaki. The only other real result is that the bacteriophages do not like the calcium in many of the buffers and only
grow well without it. Next semester, I hope to work more with the nine recovered phages from 2011, perhaps designing primers and running PCR this time. Hopefully we come up with a way to do something with the 20 phages from 2011 that were not recovered. It was proposed to use the entire DNA sample of each unrecovered phage because it is useless to us if we cannot grow it anyways, but we will continue to look into solutions. Moreover, the students in Biology 125 were able to get their plaques and the class switched to SM Buffer for Virology which had no calcium for the remainder of the year. The fact that I was able to recover a plaque for each student but one was amazing instant gratification. The results from testing the SM Buffers are the most interesting and need more research. It would be interesting to pick a plaque and resuspend it in SM Buffer and let it sit for different increments of time to see just how badly the buffer deteriorates the phage and which buffer is the least damaging out of the four variants. Furthermore, the SEA-PHAGES Program uses Phage Buffer, which is made with calcium and proved to be hard on the delicate bacteriophages. We would like to explore using Phage Buffer without calcium on more phage samples like this and see if that will help with long-term storage.