Viral Explosion: Decimating Bacteria Because We Love Them

Andrew Marcus

MBL Microbial Diversity Course Final Project

Abstract

Photobioreactors producing biodiesel has potential to supply significant amount of carbon-neutral energy to the United States. *Synechocystis* PCC 6803 is a freshwater cyanobacterium that is useful for biodiesel production because of its high lipid content. An important step in biodiesel production is lipid extraction from biomass produced in photobioreactros, but the robust membrane structure of *Synechocystis* poses a challenge for cell lysis. Researchers are seeking various physical and chemical approaches for efficient cell lysis. Cyanophage--a bacteriophage that use cyanobacteria as its host--are nature's solution to lysing cyanobacterium. Here, I borrow inspiration from the nature and propose the use of cyanophage for efficiently lysing *Synechocystis.* Today, most known cyanophages use *Synechococcus* and *Prochlorococcus* as their host, and a phage for *Synechocystis* PCC 6803 has not been discovered. Therefore, I primarily explored techniques for isolating cyanophages here. Because of the time constraints of the course (three weeks) and slowly growing *Synechocystis*, my isolation attempt for cyanophage was unsuccessful. However, I used a strain lumo15 of *Vibrio harveyi* as a model organism to demonstrate the feasibility of enriching bacteriophage from the environment. A batch reactor succeeded in at least five orders of magnitude enrichment of vibrio bacteriophages for lumo15 within 24 hours. Lumo15 exhibited differentially inhibited by different phages, ranging from complete kill to complete growth. The phages isolated with Lumo15 were able to infect other *Vibrio harveyi* species in its cluster, but not *Vibrio spp* in different genera, demonstrating a degree of host specificity. At the end, I discuss how the techniques that I learned in isolating *vibrio bacteriphages* will be useful for my future work in finding cyanophages.

Introduction

New sources of energy that avoid the release of CO_2--a major green house gas that contributes to global climate change--is necessary to meet the energy demand posed by increasing global population. Producing biodiesel using a photobioreactor is one promising approach for C-neutral fuel. In photobioreactors, photosynthetic microbes produce lipid-rich biomass by fixing CO_2 with light from the sun as the energy source. With a cyanobacterium, as an example, that has 50 % of the dry weight as lipid, photobioreactors can produce 10^7 liters of biofuels per square kilometers of area (based on Gressel, 2008). In the United States, this productivity from photobioreactors can meet the entire demand for transportation fuel by supplying biodiesel with an equivalent of 13 % of the existing croplands.

Synechocystis PCC 6803--a strain of freshwater cyanobacteria-- is well suited for large-scale production in a photobioreactor, because it is amendable to targeted genetic modifications (Vermaas, 1996). With its complete genome sequence available (Kaneko et al., 1997) and with one of the most well characterized photosynthetic system (Vermaas, et al., 2001), engineers can make rationale decision to modify its genome to produce mutants suitable for growing in photobioreactors.

An important stage of biodiesel production is the lipid extraction step that follows the biomass generation in photobioreactor. During the extraction step, *Synechocystis* cells are lysed, and the lipid fraction is separated from other materials that include proteins and polysaccharides. Often, a lipid-extraction process uses a mixture of chloroform and methanol to dissolve lipids from *Synechocystis* into the organic phase. The robust nature of the *Synechocystis* membranes can limit the total amount of lipids that the process can extract from biomass. To enhance extraction, researchers are exploring combinations of mechanical, thermal, and electrical as pretreatment options for lysing *Synechocystis* biomass.

Bacteriophage is nature's solution to bacterial cell lysis. Lytic bacteriophage tends to have rapid generation rate, which is suitable for rapid cell lysis. Many lytic phages release endolysin (a.k.a, lysozyme) during cell lysis, which cleaves peptidoglycan layer in cell wall (from gram positive bacteria) and in periplasm (for gram-negative

bacteria), leaving lipids intact. We can then extract the lipids liberated by the bacteriophage to produce biodiesel.

One major challenge in apply bacteriophage in photobioreactor is simply finding cyanophage (bacteriophage that use cyanobacteria as the host) specific to *Synechocystis.* In nature, bacteriophage can propagate only by infecting the most and by using the energy derived from the host metabolism. Because of the host dependence of bacteriophage, the abundance of bacteriophage generally correlates with the abundant of the host. In monitoring of the cyanophage for *Synechococcus* in Woods Hole, Waterbury and Volis (1993) found that the number of the cyanophage correlated directly with the number of its host with approximate lag of one month (which probably indicates the time for cynophage population to grow). Therefore, for finding cyanophage specific to *Synechocystis*, environments where *Synechocystis* is most dominant provide the best inoculums.

Although cyanobacteria in many environments can have diverse phylogeny, a few genuses of cyanobacteria establish dominance in a particular environment. *Synechococcus* is a phototrophic picoplankton--unicellular cyanobacteria with a diameter between 0.2 to 2 μm--that is ubiquitous in freshwater and marine systems (Stockner and Anita, 1986). In freshwater habitats, autotrophic picoplankton is a minor fraction of photoautorphs (< 5 %) and *Synechococcus* is the dominating picoplankton (Weisse 1993; Padisak et al. 1997). In marine environments, *Synechococcus* often competes with *Prochlorococcus*. In open ocean waters of the subtropical and tropic Atlantic and Pacific oceans, the most abundant cyanobacteria were *Synechococcus* and *Prochlorococcus* (Goericke, R. and Welschmeyer, 1993; Liu et al., 1997). The dominance of *Synechococcus* and *Prochlorococcus* in the environment, combined with sparse reporting of the presence of *Synechocystis* in the environment, suggests potential challenge for finding cyanophages for *Synechocystis.*

My original goal of my individual project was to isolate cyanophage for *Synechocystis* PCC 6803. Due to original technical difficulty growing *Synechocystis*, I ran experiments in parallel to isolate phages for strains related to *Vibrio harveyi* and *Vibrio gazogenase. Vibrio* spp. provide an idea model system for studying bacteriophage, they are fast growing and ubiquitous to marine environment, such as Woods Hole. Here,

I describe successful enrichment procedure for phage isolation. To test the host specificity, I tested the phage isolated from one strain of *V. harveyi* against other strains of *V. harveyi*, a strain of *V. fischeri*, and a strain of *V. gazogenase*. I discuss how the results apply to *Synechocystis.*

Materials and Method

Culture Information

Dr. John Waterburry from Woods Hole Oceanography Institute (WHOI) generously supplied a pure culture of *Synechocystis*. Pure cultures related to *Vibrio harveyi*, *Vibrio fischeri*, and *Vibrio gazogenes* were isolated by 2009 Microbial Diversity classmates. Table 1 summarizes the strain information. Appendix 1 summarizes the ribosomal RNA sequences for these isolates.

Table 1. Information of isolates used in this study.

Isolate ID #	**Location**	**Scientist**	**genera**	**species**
lumo2	MBL Beach	Jenna Morgan	*Vibrio*	*fischeri*
lumo5	Eel Pond	Ben Tully	*Vibrio*	*fischeri*
lumo8	Eel Pond	Terry Legg	*Vibrio*	*harveyi*
lumo9	Eel Pond	Roland Hatzenpichler	*Vibrio*	*harveyi*
lumo10	Eel Pond	Roland Hatzenpichler	*Vibrio*	*harveyi*
lumo15	Eel Pond	Teresa Kirschling	*Vibrio*	*harveyi*
goz	MBL Beach	Alex Petroff	*Vibrio*	*gazogenes*

Sampling Sites

Environmental water samples came from 8 locations near Woods Hole, MA that include freshwater, brackish water, and sea water (Table 2).

Table 2. Sampling Locations for Environmental Water

Site ID	Water Type	Location
1	Fresh	Sols Pond
2	Fresh	Intersection of W Falmouth Highway and View Crest Road
3	Fresh	Unidentified road back from little Sippewissett
4	Brackish	Sample obtained from Steve (undisclosed location)
5	Brackish	Trunk River
6	Brackish	School Street Marsh
7	Sea water	Eel Pond
8	Brackish	Goldfish Pond

Media Composition

I prepared four variants of BG-11 media growth media for Synechocystis. All media shared the following chemicals in common (in 1 liter): 1.5 g $NaNO_3$, 0.02 g Na_2CO_3, 0.001 g Na_2Mg·EDTA, 0.006 g ferric ammonium citrate, 0.006 g citric acid·H_2O, 0.036 g $CaCl_2$·$2H_2O$, 0.075 $MgSO_4$·$7H_2O$, 0.0305 g K_2PO_4, 0.00286 g H_3BO_3, 0.00181 g $MnCl_2$·$4H_2O$, 0.000222 g $ZnSO_4$·$5H_2O$, 0.000079 g $CuSO_4$·$5H_2O$, 0.0000494 g $Co(NO_3)_2$·$6H_2O$, and 0.000391 g $NaMoO_4$·$2H_2O$. One exception is Media 1, which contained 1.5 g $NaNO_2$ instead of 1.5 g $NaNO_3$. Four media used different sources of water in preparation: distilled water for Media 1 and Media 2, tap water for Media 3, water from Site 2 filtered with 0.2-μm filter for Media 4. All media had 100 mM of MOPS buffer with an initial pH of 7.0 accomplished by addition of 5 N NaOH.

Salt water complete (SWC) media contained (in 1 liter) 15 g NaCl, 1.125 g $MgCl_2$·$6H_2O$, 0.056 g $CaCl_2$·$2\ H_2O$, 0.25 g KCl, 5 g Bacto tryptone, 1 g yeast extract, and 3ml glycerol. Plates had 15 g of agar in addition. Soft agars had 0.5 to 0.6 g of agar to make approximately 0.5 % agar. All media were autoclaved upon preparation.

Growth Assay

I ran batch reactors to compare the impact of Media 2, 3, 4 on the growth rate of Synechocystis. Each batch reactor contained 50 ml of media and 1 ml of Synechocystis culture pre-grown in Media 2 to OD_{660} of 0.192. I monitored bacterial density by following OD_{600} using Genesis 20 (Thermo Spectronic). The reactors were placed next to a fluorescent light source as shown in Figure 1.

Figure 1. The experimental setup for growing of *Synechocystis.* All reactors and assay were adjacent to the fluorescent light source.

Plate Assay for Phage Enrichment

For phage isolation, I adopted 24-well tissue culture plate assay by Waterbury and Volis (1993). Each well contained a dense 0.5-ml culture of host mixed with 0.5 ml of environmental samples, which was filtered with 0.2-μm filter in order to remove bacteria, but not viruses. After 20 to 50 minutes of contact time, 1 ml of growth media was added for dilution. To detect enrichment of phage, the wells with environmental samples were compared against a control well with no environmental samples by visual inspection.

Enrichment in a Batch Reactor

For enriching bacteriophage for a specific host, I mixed 25 ml of SWC media with 25 ml of water sample from Eel pond filtered with 0.2-μm filter to remove bacterial cells, while leaving virus in the solution. The combined media was inoculated with either

Vibrio harveyi (lumo 15) or *Vibrio gazogenese* (goz) cultures pregrown in a test tube with SWC media.

DNA Isolation from Environmental Sample

Filter environment sample through 0.2-μm filter to remove cellular material. Then, filter 100 ml of filtrate onto a 0.02-μm filter to collect phage particles, then re-suspend them in 7 ml of a modified SM storage buffer (600 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris [pH = 7.5], 0.04 % gelatin).

Plaque Assay

For plaque assay 0.5 ml of water sample filtered with 0.2-μm filter was combined with 0.5 ml of a dense culture of pre-grown host bacterial cells. Upon contact time of 30 minutes, the mixture was combined with 3 ml of soft-agar at 50 ^{o}C. After moderately shaking, the mixture was poured onto a SWC plates. The plates were incubated at 30 ^{o}C.

For most probable number (MPN) assay, ten fold dilutions (from 10^{0} to 10^{-10}) of the enrichment reactor filtrate (with 0.2-μm filter) were made. 100 μl of dilutions were mixed with 900 μl of pre-grown host baterial cells. Upon contact time of 30 minutes, the mixture was combined with 3 ml of soft-agar at 50 ^{o}C. After a brief vortexing, the mixture was poured onto a SWC plates. The plates were incubated at 30 ^{o}C.

SYBR Green I Assay for Viral Counts

I adopted the protocol by Patel et al. (2007) for SYBR Green assay. 5 ml of samples were mixed with 0.3 ml of 20 % formaldehyde for crosslinking. After 1 hour of incubation, 5 ml of sample was filtered through 0.02-μm anodisc membrane filters (Whatman). After drying the filter, the membrane was air dried. When the membrane was dried, it was stained with 100 μl of 1:400 dilution of SYBR Green I. The membranes were briefly blotted against kimwipes to remove excess SYBR green. Then, the filters were again aired. Using 30 μl of 0.1 p-phenylenediamine, the membrane was mounted onto a microscope slides.

Results and Discussion

The initial microwell plate assay had Media 1 with nitrite as the nitrogen source. After three days, no observable growth of *Synechocystis* occurred in control and wells with environment filtrate, except for one well which accidently received 2 ml of Media 1 instead of 1 ml. The nitrite concentration in the Media 1 is 14.4 mM, suggesting that nitrite becomes inhibitory to *Synechocystis* between 2.4 and 3.6 mM.

Figure 2 shows one of the six microwell assays with environmental samples. The top eight columns in the first row are controls without any environmental sample and the rest of the wells are with environmental filtrate. Within 7 to 10 days of observation period suggested by Waterburry and Volis (1993), there were minor differences in colors among the wells; however, none of the wells became clear, which would have indicated the presence of lytic cyanophage. Hence, no evidence of cellular lysis was apparent from the 24-well plate assays.

Figure 2. A representative picture of two 24-well plate assays for enriching and isolate cyanophage. The top 8 columns in the first row are controls without any environmental samples. The remain wells contain environmental filtrate. No difference in colors indicates lack of bacteriophage enrichment.

To obtain fitness advantage, bacteriophages often infect exponentially growing host cells in nutrient rich environments. To test if the host cells are growing well, I constructed growth curves for *Synechocystis* in Media 1-4. Figure 3 shows the impact of

growth medium on growth curves for *Synechocystis.* Essentially no growth of *Synechocystis* occurred in Media 2 prepared with tap water, suggesting the presence of a compound toxic to *Synechocystis*. The compound is most likely chlorine, which water distribution systems add to drinking water as a disinfectant. The increase in OD_{600} in Media 3 was slightly faster than in Media 4; however, the difference is not significant, judging from the overlap in the 95-% confidence intervals. The doubling time of *Synechocystis* in media 3 and 4 were approximately 2 days, more than a factor of 2 larger than maximum doubling times for *Synechocystis* typically reported in the literature (Anderson and McIntosh, 1991), suggesting possible limitations. Researchers commonly supply *Synechocystis* culture with CO_2; these reactors obtained CO_2 passively by diffusion, hence the supply of inorganic carbon may have been limiting. Alternatively, the light intensity from a fluorescent light source (Figure 1) may have lead to photoinhibition of *Synechocystis*. Hence, one or more of these limitations may have caused *Synechocystis* host cells to grow slowly, delaying the enrichment of cyanophage in each well and making it too slow for cyanophage assay in the three-period for the course.

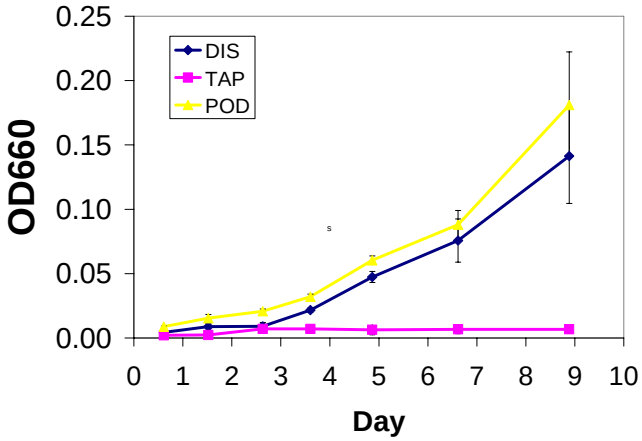

Figure 3. Growth curves for *Synechocystis* with Media 2 (DIS, blue diamond), Media 3 (TAP, pink square), and Media 4 (POD, yellow rectangle). Each point represent triplicates with an error bar showing 95-% confidence interval.

Enrichment and Characterization of *Vibrio bacteriopahge*

The tree in Figure 4 shows the phylogenetic relationship among the isolates summarized in Table 1. Four strains (lumo05, lumo09, lumo10, and lumo 14) closely clustered with *Vibrio harveyi*, with lumo15 appearing to be more distant than the other four strains. The five strain closely related to *Vibrio harveyi* clustered separately from *Allivibrio fischeri* strains and *Vibrio gazogenes*.

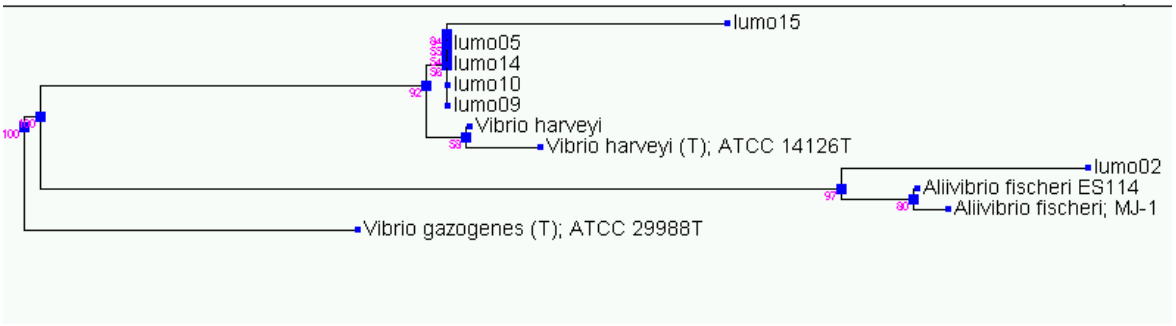

Figure 4. Phylogenic trees show the relationship among isolates summarized in Table 1 based on sequences of 16s ribosomal RNA genes. The tree was generated by tree builder method on Ribosomal Database Project website.

For initial plaque assay, I filtered the water from Eel pond with 0.2-μm filter to remove bacteria. To assay for *V. harveyi* and *V. gazogenes*, 0.5-ml portions of the filtrate was used for plaque assay. No plaque formation occurred during the assay, indicating that phages for these two *Vibrio* were not detectable with the assay.

In isolation of bacteriophages, researchers commonly concentrate environmental samples by filtration or centrifugation to artificially enrich for bacteriophages. These methods concentrate all bacteriophages in the sample with no specificity. Because I am interested in bacteriophage for *Vibrio*, I decided to run a batch reactor to enrich for host specific bacteriophage.

Figure 5 shows the plaque assay for enrichment batch reactor for bacteriophage for *V. harveyi*. The picture in Figure 4 a) was taken in the dark with 7 seconds of exposure with Panasonic Luminex digital camera. The blue light is from the bioluminescence of *V. harveyi*. After 12 hours of enrichment, the assay revealed multiple plaques on the plate (the dark points on the plate), indicating enrichment of *Vibrio*

bacteriophage. After 24 hours (Figure 4 b)), plaque assay showed that all *V. harveyi* were killed from the plate and no bioluminescence were present on the plate.

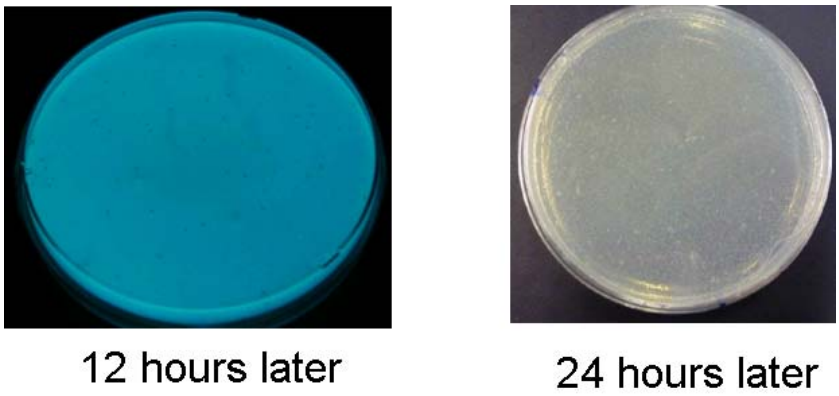

Figure 5. Plaque assay on enrichment culture for phages for a *Vibrio harveyi* strain lumo 9. After a) 12 hours and b) 24 hours of enrichments.

After 24 hours of enrichment, the plaque assay for *Vibrio gazogenes* revealed no plaques. While *Vibrio gazogenes* were isolated from MBL beach, I used the filtrate of Eel pond for enriching viruses. The lack of plaque suggests that *V. gazogenes* and its phage are less abundant in Eel pond. If those phages are present in Eel pond, longer enrichment period may enrich for *V. gazogenes* phages, which will be an excellent topic for the future studies.

Figure 6 shows the image captured by epifluorescent microscopy of vibriobacteriophage stained with SYBR Green I. The filamentous bacteria shown in the center is a bacterial cell of spirochetes, which indicates that some bacterial cells passes through 0.2 μm membranes. Cell counting of the SYBR-Green stained viruses indicate a virus count of 1 (± 0.7) 10^{10} cells per ml of enrichment sample. The high error in the m occurred likely because the membrane was wet due to short drying time; this free some viruses mobile, as it was observed in the microscopy, making the distribution of viruses on the membrane inhomogeneous. In agreement with the measurements from microscopy, quantification with the MPN assay indicated virus count of > 10^{10} bacteriophage per ml. Because a typical total virus counts in marine environment is

about 10^6 viruses per ml, the MPN and microscopy results indicate that there is at least 10^5 fold concentration of *Vibrio* specific phage because of the enrichment in batch.

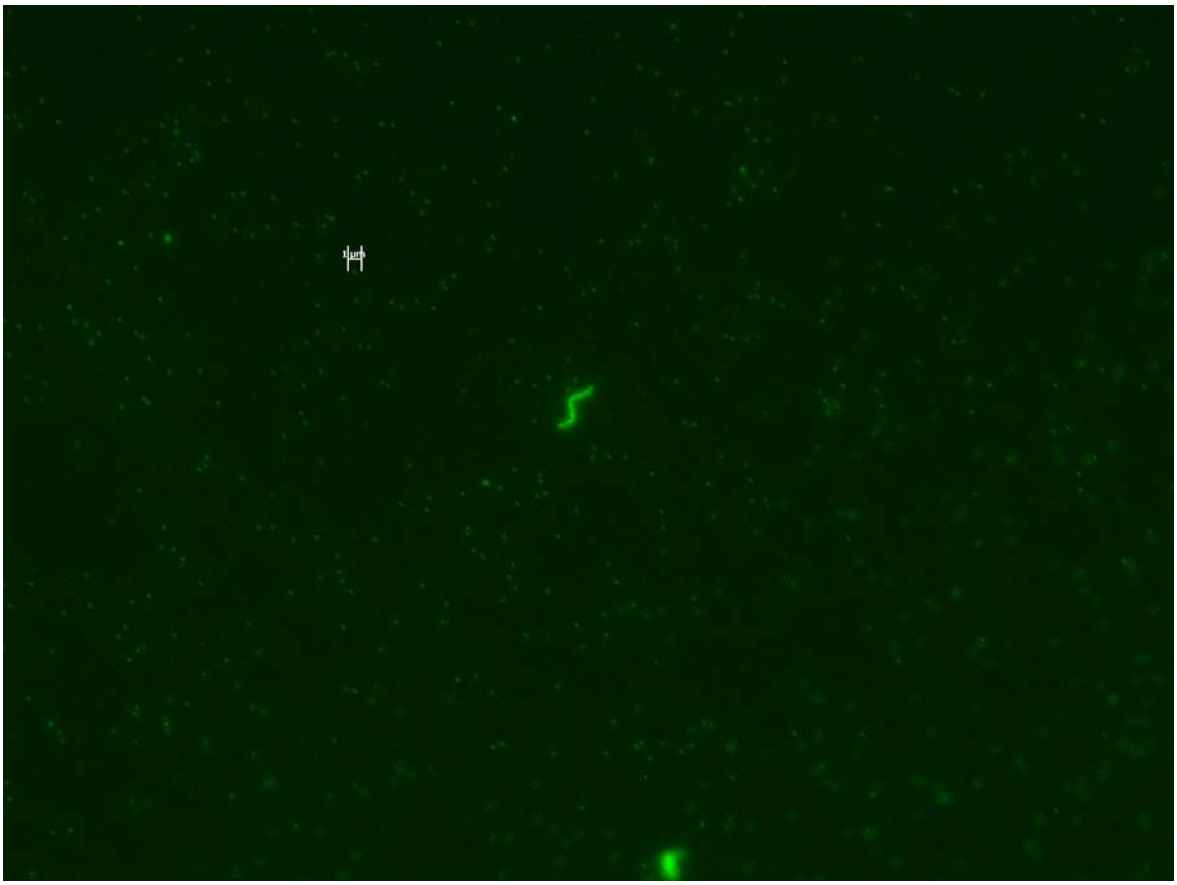

Figure 6. Epifluorescent microscopy image of *vibrio bacteriophages* filtered onto a 0.02 μm filter and stained with SYBR Green I. The image is with 100 X oil emersion lens.

In order to isolate phage specific to vibrio from the enrichment, I performed assay in 96-well plate. In row 2, *Vibrio* strain lumo15 was placed in each column. The column with control received no viruses. Each well received a different plaque from plaque assay (e.g., Figure 5 a) picked with a sterile tooth pick. After 12 and 24 hours of incubation at 30 ^{o}C, only the control well turned white, indicating growth (Figure 7). After 31 hours, wells 1, 3, 4, 5, and 10 showed slight white coloration, indicating hindered growth and well 11 turned completely white. Hindered growth suggests the presence of *Vibrio* strains resistance to viral infection. After 45 hours, most of all wells but well 8 had complete growth. The phages from transferred from row 2 to the wells directly below in row 3 with fresh *Vibrio harveyi* strain lumo15. Well 8 was completely

cleared and well 6 had hindered growth. Thus, bacteriophage has differential impacts of on the host ranging from complete kill, delayed hindered growth, and delayed, but, complete growth.

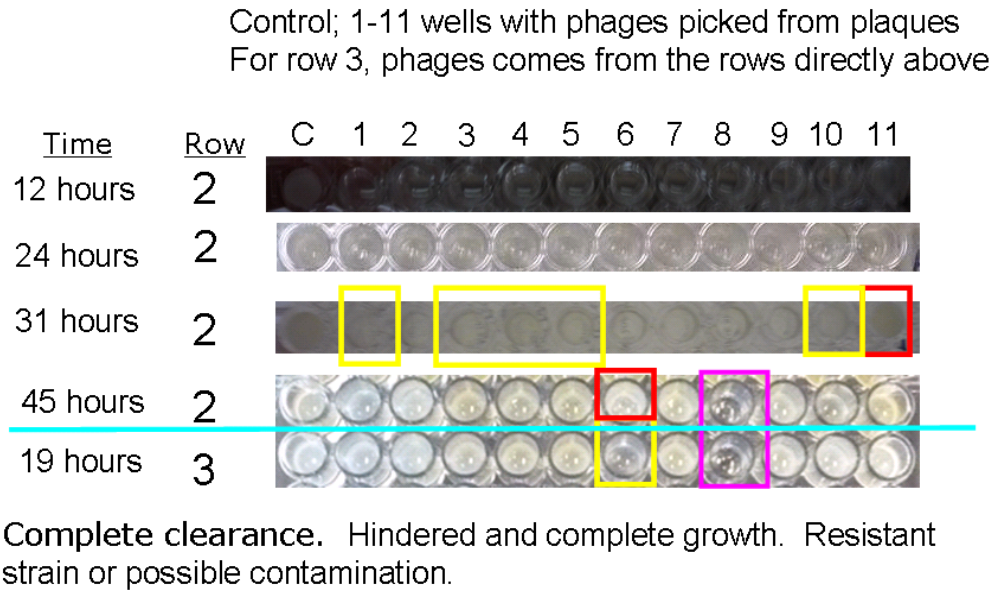

Figure 7. Isolation of vibrio bacteriophage in 96-well plate. Column 1 are control wells with no phage. Columns labeled 1 to 11 each has different plaque from the plaque assay picked with a sterile toothpick. The results for well 2 are shown at four time points (12, 24, 31, and 45 hours) and well 3 is for one time point (19 hours).

Because the phages from wells 6 and 8 exhibited the strongest inhibitions, they were used in the host specificity of with isolates from Table 1. Figure 8 shows the result of host specificity test after 12 hours of incubation. Llumos 2, 5, 8, and 14 and *Vibrio gazogenes strain goz* (R and P) were unaffected by the phages. The growth of lumos 9 and 10 were hindered by phages 6 and 8. For lumo15, bacteriophage from column 6 hindered growth while phage 8 completely inhibited the growth. Examination of the phylogenic tree in lumo9 and 10, which showed similar response are clustered together. Furthermore, the strains affected by the virus were all from the genera Vibrio harveyi. Strains related to *V. fischeri* and *V. gazogenes* were unaffected. The result from very limited sampling indicates that specificity of these *Vibrio* is limited to those strain clustered with *V. harveyi*. Experiments with more strains of *Vibrio* are needed to more carefully define the specificity.

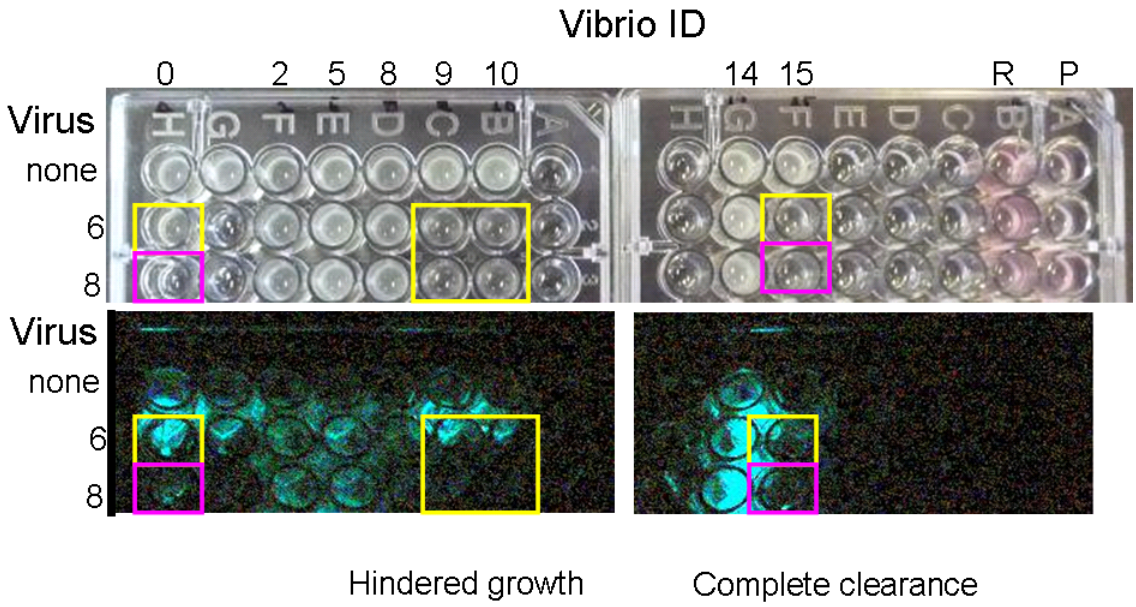

Figure 7. The test of host specificity by test testing the phages for lumo15 on laboratory isolates summarized in Table 1. The photograph is taken after 12 hours of incubation. The top image of microwell is taken in the light; the second image is taken in the dark.

Conclusion and Future Work

The enrichment of vibrio phage for strains of *Vibrio harveyi* was successful. The assays on 96-well plate illustrated differential impacts of phages on a host, specificity of phages towards hosts, and possible clustering of host that are susceptible to a particular phage. These assays were limited in scope and the study should be expanded to refine the conclusion, especially by considering more strains of *Vibrio spp.* and by isolating more phages.

During the three week enrichment period, cyanophage for *Synechocystis* or bacteriophage for a strain of *Vibrio gazogenes* could not be enriched. The cyanophage enrichment was confounded largely by the slow growth rate of *Synechocystis.* For future work, effort to optimize the growth of *Synechocystis* in both broth and plates is necessary.

The lack of vibrio phages for a strain of *V. gazogenes* was a surprise, since the strain was isolated in Woods Hole, and I expected that its phage will be present in the host's native habitat. It is possible for *V. gazogenes* to be resistant to phage. However, since the gazogenes strain came from the MBL beach, instead of the Eel pond, the initial

inoculum may have been poor. Phages for *V. gazogenes* may be more abundant in the MBL water samples.

Short enrichment period of 24 hours was one major shortcomings of the enrichment protocol, which may not have been sufficient for vibrio bacteriophage to become enriched. In earlier studies for *Synechococcus* in open oceans, Water and Volis (1993) observed that the trends cyanophage number correlated, but lagged behind the host's trend by about 1 month. Therefore, more prolonged enrichment period maybe necessary before phages become detectable by plaque assay. A simple extrapolation based on the doubling time (< 1 hour for *Vibrio harveyi* and 2 days for *Synechocystis*) suggest that enrichment period needed to be 2 to 4 weeks long. In this work, I was able to successfully apply SYBR Green assay for directly counting viruses by epifluorescent microscopy. For pro-longed enrichment period, SYBR Green assay may become useful to observe slow enrichment of cyanophages, which may not be been detected by plaque assay.

I plan to continue working on the enrichment of cyanophage for *Synechocystis* and on the study of *Vibrio harveyi* strain upon my return to Arizona State University. For *Synechocystis*, I plan to use filtered water samples from Sites 1, 2, and 7 as the initial inoculum sources for phages. In addition to these environmental samples, I will monitor the effluent from the photobioreactors to see if bacteriophage are abundant.

There are several important questions:

1. Given the high abundance of *Synechococcus* in the environment, how prevelant are cyanophage for *Synechocystis*.
2. Provided with enough time, can cyanophage for other host such as *Synechococcus*, adapt to infect *Synechocystis*?
3. What are the characteristics of the cyanophage that makes them very efficient for cellular lysis?
4. How does the cyanophage influence nutrient cycling during cellular lysis? In photobioreactors? Can cyanophage increase primary productivity in the photobioreactors by promoting nutrient cycling?

For answering these questions, the establishment of enrichment protocol is necessary.

Meanwhile, there is a need for establishing engineering principles of operating a phage enrichment and biomass lysing reactor. *Vibrio harveyi* and its phage is an excellent model system to work out the principle, because of their fast growth rate. Therefore, I plan to work on *Vibrio harveyi* to understand the kinetics and dynamics of phage production and bacterial cell lysis.

References

Anderson S, McIntosh L. 1991. Light-activated heterotrophic growth of the cyanobacterium Synechocystis sp strain PCC-6803 - A blue-light-requiring process. J Bacteriol 173(9):2761-2767.

Goericke R, Welschmeyer N. 1993. The marine prochlorophyte Prochlorococcus contributes significantly to phytoplankton biomass and primary production in the Sargasso Sea. DEEP-SEA RESEARCH PART I-OCEANOGRAPHIC RESEARCH PAPERS 40(11-12):2283-2294.

Gressel J. 2008. Transgenics are imperative for biofuel crops. Plant Science 174(3):246-263.

Kaneko T, Tabata S. 1997. Complete genome structure of the unicellular cyanobacterium Synechocystis sp. PCC6803. Plant Cell Physiol 38(11):1171-6.

Liu H, Nolla H, Campbell L. 1997. Prochlorococcus growth rate and contribution to primary production in the equatorial and subtropical North Pacific Ocean. AQUATIC MICROBIAL ECOLOGY 12(1):39-47.

Padisak J, Krienitz L, Koschel R, Nedoma J. 1997. Deep-layer autotrophic picoplankton maximum in the oligotrophic Lake Stechlin, Germany: origin, activity, development and erosion. Eur J Phycol 32:403-416.

Patel A, Noble R, Steele J, Schwalbach M, Hewson I, Fuhrman J. 2007. Virus and prokaryote enumeration from planktonic aquatic environments by epifluorescence microscopy with SYBR Green I. Nature Protocols 2(2):269-276.

Stockner J, Antia N. 1986. Algal picoplankton from marine and freshwater ecosystems: a multidisciplinary perspective. Can J Fish Aquat Sci 43(12):2472-2503.

Vermaas W. 1996. Molecular genetics of the cyanobacteriumSynechocystis sp. PCC 6803: Principles and possible biotechnology applications. J Appl Phycol 8(4-5):263-273.

Waterbury J, Valois F. 1993. Resistance to Co-Occurring Phages Enables Marine Synechococcus Communities To Coexist with Cyanophages Abundant in Seawater. Appl Environ Microbiol 59(10):3393-3399.

Weisse T. 1993. Dynamics of autotrophic picoplankton in marine and freshwater ecosystems. 13:327-370.

Appendix 1. The DNA sequences for 16sRNA sequence for the environmental isolates used in this study.

>lumo02

TGCAGTCGAGCGGAACGACTTAACTGAACCTTCGGGGAACGTTAAGGGCGTCGAGCGGC
GGACGGGTGAGTAATGCCTGGGAATATGCCTTAGTGTGGGGGATAACTATTGGAAACGA
TAGCTAATACCGCATAATGTCTTCGGACCAAAGAGGGGGACCTTCGGGCCTCTCGCGCT
AAGATTAGCCCAGGTGAGATTAGCTAGTTGGTGAGGTAAGAGCTCACCAAGGCGACGAT
CTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCC
GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTAGGGAGGAAGGTGTTGT
AGTTAATAGCTGCAGCATTTGACGTTACCTACAGAAGAAGCACCGGCTAACTCCGTGCC
AGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ATGCAGGTGGTTCATTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACCGCATT
TGAAACTGGTGAACTAGAGTGCTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAA
TGCGTAGAGATCTGAAGGAATACCAGTGGCGAAGGCGGCCCCCTGGACAGACACTGACA
CTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGTCTACTTGGAGGTTGTTCCCTTGAGGAGTG

>lumo05

CTACCATGCAGTCGAGCGGAACGACTTAACTGAACCTTCGGGGAACGTTAAGGGCGTCG
AGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGG
AAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCT
CGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGC
GACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGC
CATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGG
TGGTGGTGTTAATAGCACTATCATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTC
CGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTA
AAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAAT
AGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCG
GTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAA

>lumo09

TGCAGTCGAGCGGAACGACTTAACTGAACCTTCGGGGAACGTTAAGGGCGTCGAGCGGC
GGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGA
TGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTC
AGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGAT
CCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC
GCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTGGTAG
TGTTAATAGCACTATCATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCC
AGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATT
TGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAA
TGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACA
CTCAGATGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AACGATGTCTACTTGGAG

>lumo10

TGCAGTCGAGCGGAACGACTTAACTGAACCTTCGGGGAACGTTAAGGGCGTCGAGCGGC
GGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGA
TGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTC
AGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGAT
CCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC
GCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTGGTAG
TGTTAATAGCACTATCATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCC
AGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATT
TGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAA
TGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACA
CTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
ACGATGTCTACTT

>lumo14

TGCAGTCGAGCGGAACGACTTAACTGAACCTTCGGGGAACGTTAAGGGCGTCGAGCGGC
GGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGA

TGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTC
AGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGAT
CCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC
GCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTGGTGT
AGTTAATAGCACTATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCC
AGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATT
TGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAA
TGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACA
CTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT

>lumo15

TGCAGTCGAGCGGAACGACTTAACTGAACCTTCGGGGAACGTTAAGGGCGTCGAGCGGC
GGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGA
TGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTC
AGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGAT
CCCTAGCTGGTCTGAGAGGATGATCACCCACACTGGAACTGAAACACCGTCCAGACTCC
TACGGGAGGC