

ϕFISH - a method for FISHing bacteriophage host

Gil Sharon

Dept. of Molecular Microbiology and Biotechnology, Tel Aviv University, Tel Aviv, 69978.

gilsharo@post.tau.ac.il.

Abstract

Viruses, the most abundant biological entities in the oceans, have a significant role in driving the microbial loop by making particulate organic matter and dissolved organic matter from photoautotrophs and heterotrophs (Suttle 2007). This mini-project aimed at devising a method for identifying hosts of bacteriophages (phages) by FISH probing. Probes can be constructed from the phage DNA (or a PCR product, if only a sequence is available) that is restricted and labeled. Later, samples can be sorted out of the sample in order to probe 16S rRNA genes present to determine host range.

Even though results were not as satisfying as expected, it does look like this method is in the right way. More calibrations of the method should be sufficient in order to prove this method as a useful method for pinpointing phage host range.

Introduction

Viruses are the most abundant biological entities in the oceans, comprising ca. 94% of the DNA/RNA-containing particles (Suttle, 2007). Viruses have a significant role in driving the microbial loop by making particulate organic matter and dissolved organic matter from photoautotrophs and heterotrophs (Suttle 2007).

In recent years a number of groundbreaking studies on viral metagenomics opened the door to this fascinating world. However, even though genomic data is available, little progress has been made towards identification of the hosts for these viruses.

This mini-project aimed at devising a method for identifying hosts of bacteriophages (phages) by FISH probing. For the sake of this exercise, let's say we have an entire viral genome and we're looking for its host. By designing specific primers, the genome of this virus (and maybe other very closely related ones) can be picked up by PCR. Once we have the DNA, it can be restricted for probe preparation (preferably with a blunt end cutter, but not necessarily). After restriction, 150-350 bp fragments are purified from the gel (restriction enzyme choice is made according to the coverage you get by 150 – 350 bp fragments. Should aim at >5000bp). Future-Probe fragments are then labeled fluorescently using either nick-translation or other labeling kits and cleaned up from any leftovers. Fixed and permeabilized samples are filtered onto a 0.2 μm (or kept in solution) and hybridized according to the geneFISH protocol (Moraru et al, 2010). Later, samples can be sorted using FACS or catapulted from the membrane using PALM and a clone library will be constructed in order to identify.

In essence, I aimed at proving the concept that a phage's host range can be identified straight from an environmental sample and the virus' genome sequence (even partial). I tried to do so by using an isolated phage and its host (both isolated by Alison Ling), though I had no sequence information.

Materials and Methods

Bacteriophage and bacterial host

An alpha proteobacterium hereon be referred to as AliBug (identified as a *Pseudoalteromonas* sp. by 16S rRNA gene sequencing) isolated from Eel pond (Woods Hole, MA) and its bacteriophage (hereon referred to as AliPhage) were both isolated by Alison Ling (See miniproject report) - .

Lysate preparation, Phage isolation and DNA extraction

All phage-related methods were devised based on protocols of lambda phage in Molecular Cloning: a laboratory manual (Sambrook and Russel, 2001). Lysates were prepared by diluting an overnight culture of AliBug 1:100 in SWC, growing it to about 10^7 - 10^8 cells/ml (late logarithmic stage). At that time, an aliquot of AliPhage was added to the growing culture and monitored until the culture cleared. Upon clearing the lysate was centrifuged and filtered through a 0.2 μ m filter, keeping the flow through. The flow through was then treated with DNAase I and RNAase A (at a final concentration of 1 μ g/ml) incubated at room temperature for 30' and then brought to 1M NaCl and 10% PEG 8000, incubated for >1 h at 4°C and centrifuged 5000 x g, 15' at 4°C. the pellet was then resuspended in SM buffer and PEG was removed by chloroform, keeping the aqueous phase. The cleaned phage was then loaded onto a CsCl gradient with 1.45-1.75 g/ml densities. This gradient was centrifuged in an ultracentrifuge at 30,000xg for 2 h and the apparent band of phage particles was extracted using a syringe. Isolated AliPhages DNA was then extracted using a standard phenol:chloroform extraction and DNA precipitation.

DNA restriction, gel extraction and labeling

AliPhage DNA was restricted using RsaI (according to the manufacturer's specifications), loaded on a 2% agarose gel and visualized using EtBr. Bands corresponding to 150 – 350 bp fragments were extracted from the gel and DNA was purified using Qiagen gel extraction kit (according to the manufacturer's specifications). Since DNA amount was very low at this stage, an MDA reaction was setup to try and increase amount of DNA (Qiagen REPLI-g kit) and cleaned of any <70 bp fragments using Micro Bio-Spin 30 (BIO-RAD). After MDA, the amount of DNA increased to 50 ng/ μ l. The DNA was then precipitated and concentrated x20 and subsequently

used for ULYSIS (invitrogen) labeling with Alexa fluor 488. The probe was then cleaned on NucAway Spin Columns (Ambion). The labeling efficiency was then calculated and found to be 1/198.6 fluorophore/bases, hence, most probes have a single label on them (a rather low labeling efficiency).

AliPhage - One step growth curve

A one-step growth curve was done at an MOI=0.1. After allowing attachment (15', room temperature, no shaking) the culture was centrifuges and the bacteria were washed to remove all free phage from the culture. Then a sample was taken every 10' for 1 hour, fixed in 1% PFA and filtered onto a 0.2 µm filter. In addition, two control filters were made, one with uninfected *E.coli* and the other with uninfected AliBug.

Fluorescent in situ Hybridization

First the filters were embedded in agarose 0.1% (methaphore), permeabilized using 20 µg/ml lysozyme for 1 h at 37°C. Prehybridization was done by incubation with hybridization buffer (see geneFISH protocol, Moraru et al, 2010) for 1 h at 75°C, and then probe was added to a final concentration of 5 ng/ml and incubated for 5 h at 46°C. the filters were then washed 3 times in 2xSSC, 0.1% SDS in PBS at 48°C for 10 minutes. Then washed for 1 h in 0.1% SSC, 0.1% SDS in PBS at 48°C. the filters were then washed in MilliQ water for 1 minute, 96% ethanol and air dried. Finally, the filters were then mounted in Citifluor with DAPI. Samples were visualized with a ZEISS epifluorescent microscope.

Results

Figures 1 and 2 show results of the ϕ FISH. Controls (Fig. 1) show weak signal, mostly. The hybridization with AliPhage infected cultures did show few strong signals, above background (Fig. 2).

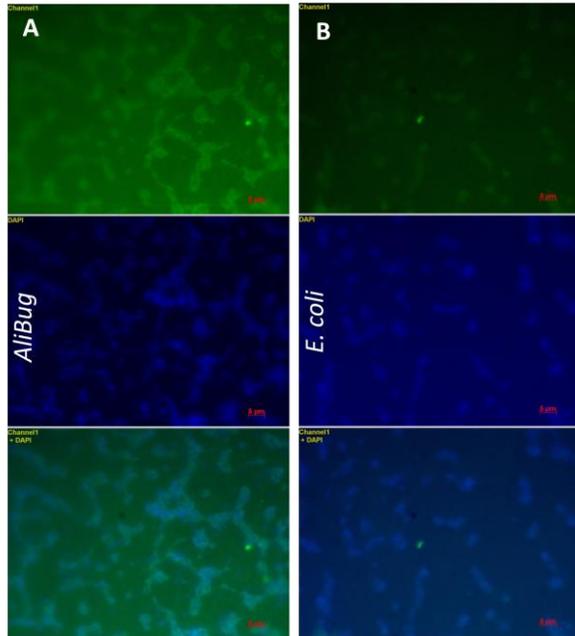


Figure 1. ϕ FISH controls, hybridization to (A) AliBug and (B) *E. coli*. Upper panel – Alexa fluor 488, middle panel – DAPI and lower panel – merged.

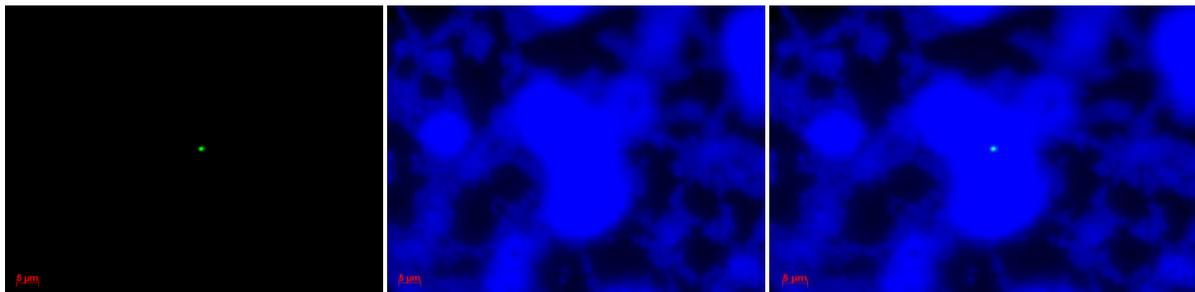


Figure 2. ϕ FISH on 40' post attachment sample. Left panel – Alexa fluor 488, middle panel – DAPI and right panel – merged.

Discussion

This project tried to provide a proof of concept for a method to identify a phage's host range straight from an environmental sample and the virus' genome sequence or an isolate. I tried to do so by using an isolated phage and its host, though I had no sequence information. In addition an attempt to do the same with lambda phage was unsuccessful (during the probe preparation).

Results obtained here were insufficient for proof of concept. Since only a single trial was done, with one set of hybridization conditions (and without knowing the average G+C content of the probes) it was not possible to get good hybridization. In addition, the advantage of using lambda phage in order to check hybridization to prophage as well as to active infections wasn't accomplished when using AliPhage. However, it does show that hybridization to bacteria during active phage infection is possible. It is also possible that the results were not satisfying due to very low MOI or host resistance, since there was no substantial increase in the number of active phage infection-labeled cells.

The next step, which I did not get to, is to try and sort these cells out or to use PALM to catapult them out and construct clone libraries for host range identification. This should be possible due to the fact that phages usually breakdown host DNA, to serve as building blocks for their own DNA replication. But, bacteriophages usually use the host's ribosomes where there should be an intact copy of the 16S rRNA to be used for host identification. In case the infection it fixed at an early stage or the infection, it should still be possible to get the 16S rRNA gene. And in case of a prophage (if indeed it can be identified using this method), it should not be a problem. It should also be considered that the geneFISH protocol might also be useful for this purpose, with possibly more specificity and stronger signal, due to the CARD step.

Acknowledgments

I would like to thank Cristina and Ali for great help with FISH and phage work. I would also like to thank Ilil Atad, at the Tel Aviv University for the Lambda Phage, and Prof. Eugene Rosenberg and Prof. Daniel Segal. I would also like to thank everyone who offered support, advice, help, a shoulder, phage, caffeine, pizza, helpful and interesting discussions and translations to foreign

languages (what what?). Or in other words, I would like to thank the directors, the TAs, the course coordinator and the class of 2010. This unique experience would not have been possible without the support of The Katzir-Katchalsky Student Travel Fellowship, The Joan and Jaime Constantiner Institute of Molecular Genetics travel scholarship, The Herbert W. Rand Fellowship and Scholarship fund and The Moshe Shilo Memorial Scholarship Fund. Last, but certainly not least, I would like to thank Narwhals (*Monodon monoceros*), just for being there.

References

- Moraru C, Lam P, Fuchs BM, Kuypers MMM, & Amann R (2010) GeneFISH - an in situ technique for linking gene presence and cell identity in environmental microorganisms. *Environmental Microbiology* (Epub before print).
- Suttle CA (2007) Marine viruses - major players in the global ecosystem. *Nature Reviews Microbiology* 5:801-812.