Searching for novel giant viruses in the unexplored protist hosts *Corallomyxa tenera* and *Oxyrrhis marina*

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Abstract

Viruses play major roles in microbial systems as predators, potentially impact global processes (*e.g.* the carbon cycle), and represent the most underexplored source of nucleic acid diversity. The order *Megavirales*, comprised of the giant viruses, potentially includes a large amount of diversity, as these viruses encode large (0.3-1.3 Mb) genomes. Most known giant viruses have been isolated in the amoeba *Acanthamoeba*, introducing a potentially severe sampling bias. This project uses *Oxyrrhis marina* and *Corallomyxa tenera* as unexplored hosts of giant viruses. Viruses were enriched from environmental samples through passaging size-fractionated culture supernatants. Those enriched viral fractions were sequenced (pending results), imaged by TEM, and hosts were reinfected and imaged by fluorescence *in situ* hybridization microscopy. Although results are unclear, as no immediately obvious giant virus particles were identified, at least one reinfection culture displays a phenotype in the host. Further work will be needed to refine these techniques to yield more informative results.

Background

Viruses are ubiquitous in all ecosystems, infecting all living things. As predators of microbial hosts, viruses likely play roles in global processes dominated by microbial systems (*e.g.* the carbon cycle). In addition to their ecological impacts, viruses are evolutionarily important as vectors for horizontal gene transfer between hosts, and as rapidly mutating genomes representative of immense sequence diversity.

Giant viruses, belonging to the viral order *Megavirales*, were only recently discovered, having been overlooked by traditional methods for viral purification that presume that all viruses must be small enough to be isolated through a 0.22 μm filter (1). Giant viruses are protist-associated dsDNA viruses, and replicate in both the host nucleus and cytoplasm. They are united by their large particle sizes (300-600 nm diameter), large genome sizes (0.3-1.3 Mb), and large complement of genes for DNA repair, DNA replication, transcription, and translation (2). Most giant viruses have been isolated in the free-living amoeba *Acanthamoeba*, an easily culturable host (2). Few other giant viruses have been described in diverse hosts: the flagellate *Cafeteria* (3), the algae *Tetraselmis* (4), and another amoeba *Vermaboeba* (5). This suggests giant viruses are ubiquitous in protist hosts, though much of this viral diversity likely remains unexplored due to a lack of using novel host systems.

The aim of this project is to enrich for giant viruses using the hosts *O. marina* (Fig. 1A), a dinoflagellate of the Alveolata superphylum, and *C. tenera* (Fig. 1B), an amoeba of the supergroup Rhizaria. These hosts were chosen to expand the range of protist hosts that have

been used for new giant virus discovery. No viruses infecting either of these hosts are known and no giant viruses have been described for any hosts in the large protist clades Alveolata or Rhizaria. It was hypothesized that giant viruses infecting these protist hosts, *O. marina* and *C. tenera*, could be isolated from their respective environments, given the wide host range of giant viruses across the eukaryotic tree of life.

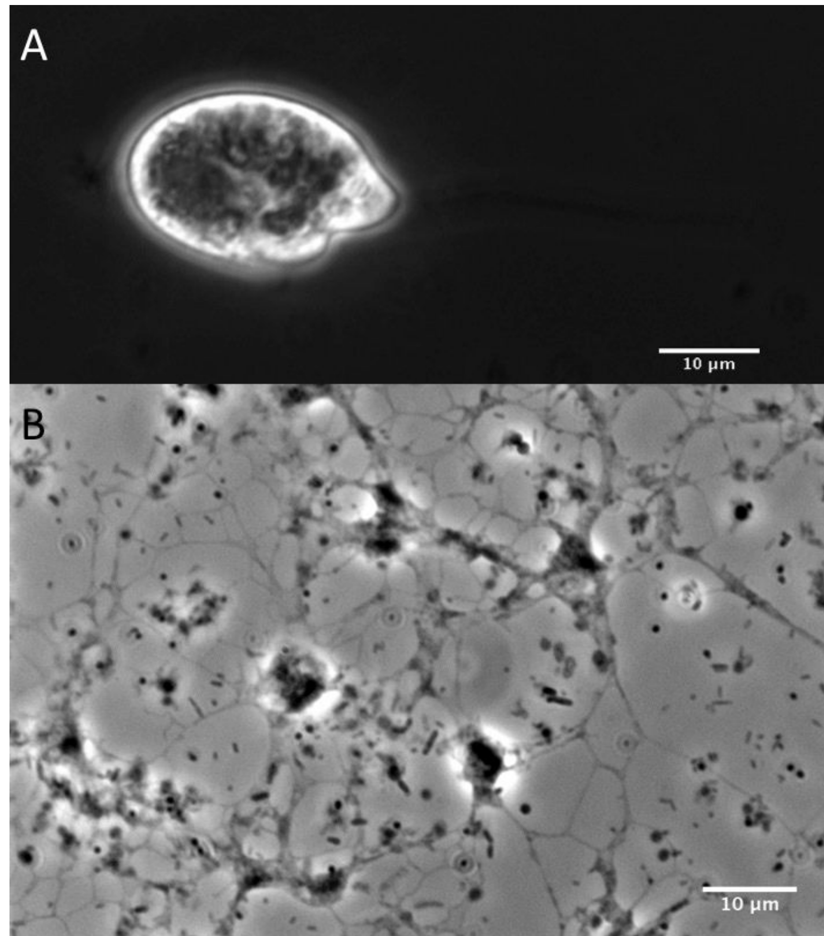

Figure 1. Protist hosts used for enrichment of giant viruses in this study: (A) the dinoflagellate *O. marina*, and (B) the amoeboid Rhizarian *C. tenera*.

Methods

Environmental Sampling & Preparation

Samples were collected from each of the protist hosts' habitats. For *C. tenera*, 1 L of water was collected from Little Sippewissett Salt Marsh after resuspending the top layers of sediment. This sample (2018.DC_29.07.01) was taken from an area of the marsh where the waters were relatively still, and sediment was loose sand with no apparent microbial mats. For *O. marina*, 1 L of surface seawater was collected from Woodneck Beach, where the water is a depth of about 3 ft (2018.DC_29.07.02).

Both samples were held at 4°C for 24 hr, then pre-filtered through a series of polyethersulfone filters: 500 µm, 120 µm, 45 µm, 7 µm. Following pre-filtration, both were passed through a 0.22 µm polyvinylidine fluoride filter. The flow-through was discarded, and filters were placed in sterile Petri dishes with 15 mL 0.5X SWB, stored at 4°C for 24 hr, and shaken on an orbital mixer for 1 hr to allow viruses to desorb from the membrane. These liquids were collected and stored at 4°C, and used as the viral samples for enrichment.

Culture Conditions

C. tenera was originally isolated from sediment samples taken from Little Sippewissett Salt Marsh in 2014, and obtained from the Dawson Lab as a coculture with *Maribacter* as bacterial prey. *C. tenera* was cultured in 0.5X saltwater base (SWB) with 5 mM MOPS buffer. Yeast extract was added every 48 hours to a final concentration of 0.01% to maintain growth of prey cells. All cultures were incubated at room temperature in 75 cm^2 cell culture flasks in 50 mL volumes. To start new cultures, 1 part dense starter culture was added to 49 parts fresh medium.

O. marina sp. was isolated from the Caribbean Sea and obtained from the Marine Biological Laboratory Physiology Course as a mixed culture containing choanoflagellates and a bacterial consortium. *O. marina* was cultured using the same methods and medium as *C. tenera*.

Viral Enrichment

Cultures of each host organism were started in 50 mL volumes 48 hr prior to addition of the virus sample, to allow for hosts to grow to high densities. Each host enrichment was performed in triplicate, using the same filtered environmental viral inoculum. After 48 hr, 25 mL of each culture supernatant was passed through a 0.22 µm polyvinylidine fluoride filter. The flow-through was discarded, each filter was placed in a sterile Petri dish with 5 mL 0.5X SWB, and shaken on an orbital mixer for 1 hr to allow viruses to desorb from the membrane. This liquid was collected and inoculated in a fresh host culture. Each enrichment was passaged twice in this manner.

Viral Purification

After viral enrichment, the 50 mL of each culture's supernatant was passed through 0.8 µm polycarbonate filters, size selecting against large cells. This filtrate was passed through a 0.22 µm polyvinylidine fluoride filter. The flow-through was discarded, each filter was placed in a sterile Petri dish with 5 mL 0.5X SWB, and shaken on an orbital mixer for 1 hr to allow viruses to desorb from the membrane. To select against persisting cells, virus samples were incubated at room temperature for 24 hr in 1 mg/mL ampicillin, 1 mg/mL cycloheximide, 1 mg/mL rifampicin, 20 µg/mL tetracycline, and 0.1 mg/mL lysozyme. To remove antibiotics, samples were pelleted at 12,000 RPM for 10 min and washed three times in 0.5X SWB. Virus samples were incubated at room temperature for 12 hr in 2 mg/mL lysozyme and 6U/µL DNase I to remove as much contaminating DNA as possible, pelleted at 12,000 RPM for 10 min and washed three times in 0.5X SWB.

Viral DNA Isolation & Sequencing

First 3U proteinase K per 100 µL sample were added to remove DNase enzyme and begin degradation of viral capsids. Following 2 hr incubation at room temperature, SDS was added to a final concentration of 2% and incubated for 24 hr at room temperature. DNA was purified from these samples using a Qiagen PowerFecal DNA Kit and quantified with a Qubit High Sensitivity dsDNA Assay Kit. Each DNA sample was double barcoded individually and prepared for sequencing with an Illumina Nextera XT kit, to be submitted for sequencing on an Illumina HiSeq 4000 (results pending; will be received after the end of the course).

Epifluorescence Microscopy

Viral samples were stained with 1X SYBR Gold and incubated at 4°C 35 min. After staining, samples were adsorbed on 0.2 µm polycarbonate filters using a vacuum manifold. Filters were fixed with ProLong Diamond Antifade mountant. Viral particles were visualized on a fluorescence microscope under 1000X magnification.

Viral Infection

To 9 parts of dense cultures of each protist host, 1 part of each viral enrichment was added. For *C. tenera*, cultures were grown with a poly-L-lysine-coated coverslip, such that adhered cells could be readily imaged. These cultures were maintained for 4 days prior to imaging.

Fluorescence *In Situ* Hybridization

Fixation of *C. tenera* was achieved using a protocol obtain from the Dawson lab. Briefly, coverslips were washed three times in calcium-free seawater before fixation for 30 sec in a 0.5% glutaraldehyde solution. After fixation, rehydration in 50 mM PIPES, 0.5 mM EGTA, 0.05 mM $MgSO_4$, 0.5% BSA, 0.05% NaN_3, 50 mM lysine, and 0.025% gelatine (PEMBALG) buffer was allowed to occur for at least 1 min, and coverslips were washed twice in PEMBALG. Permeabilization was done with 0.1% Triton-X in PEMBALG for 10 min, then washed three times in PEMBALG. Quenching of autofluorescence was accomplished with a 1 hr incubation in 0.5 M glycine in PEMBALG before three washes in PEMBALG. Fixed coverslips were kept at 4°C in PEMBALG until further use.

Fixation of *O. marina* was attempted with 2% paraformaldehyde for 8 hr at 4°C, and with 1% paraformaldehyde for 30 min. Fixed cells were immediately dried on gelatin-coated slides.

To visualize bacterial cells, 5 ng/µL EUB338-Cy3 FISH probe was made in hybridization buffer (0.9 M NaCl, 0.02 M Tris-HCl, 35% formamide, 0.01% SDS), and flooded on samples fixed to coverslips or slides. Hybridization was carried out for 1.5-3 hr at 46°C. Samples were washed for 15 min at 48°C in washing buffer (0.07 M NaCl, 0.02 M Tris-HCl, 5 mM EDTA, 0.01% SDS) and rinsed in MilliQ water. To counterstain, 1 µg/mL DAPI solution was flooded over samples for 3 min, and slides were rinsed in MilliQ water. Because *C. tenera* is sensitive to desiccation, slides were allowed to dry slightly before being mounted in ProLong Diamond Antifade mountant.

Transmission Electron Microscopy

Enriched virus samples were adsorbed to formvar coated 200 mesh copper EM grids and stained with 1% uranyl acetate prior to imaging.

Results & Discussion

Tracking giant viruses during enrichment

Tracking of giant viruses was accomplished with epifluorescence microscopy (Fig. 2). These fractions still contained large amounts of bacterial cells, which obscured imaging. Viral fractions were adhered to 0.2 μm filters, so as to focus on larger particles.

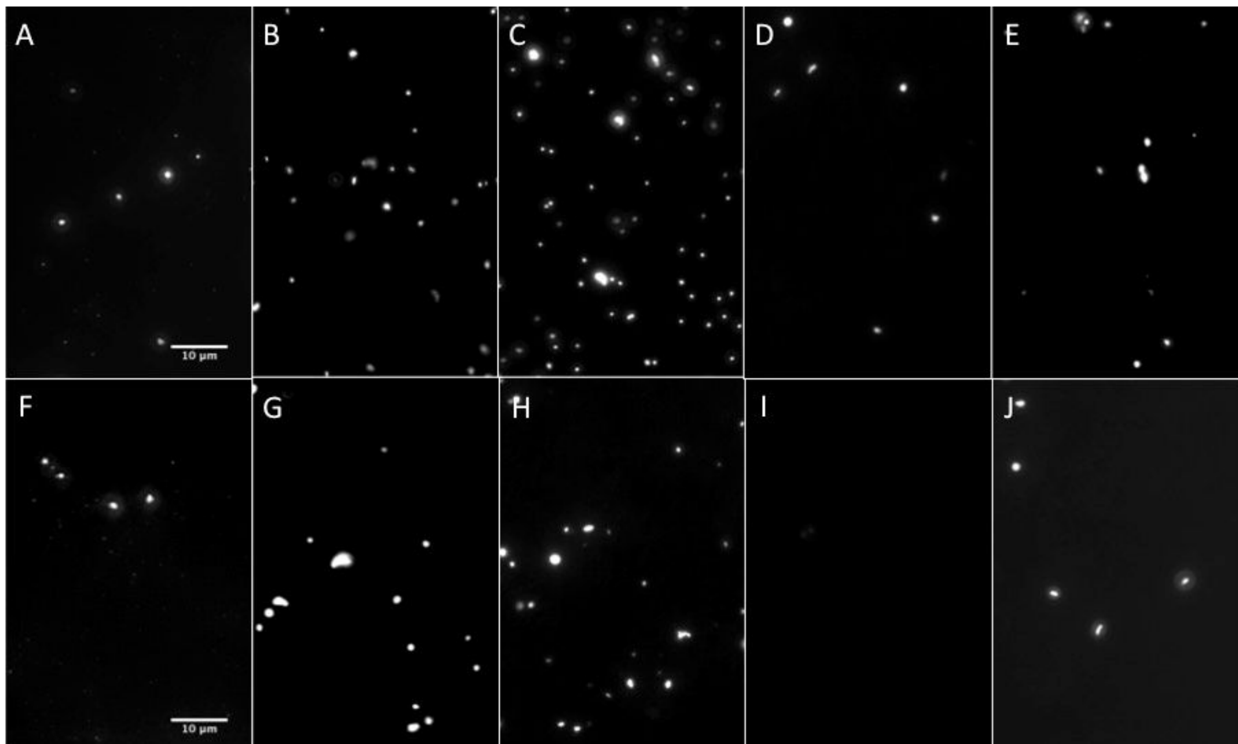

Figure 2. Epifluorescence microscopy of viral fractions during enrichment: (A) viral inoculum from Sippewissett Salt Marsh before enrichment on *O. marina*, (B) *O. marina* uninfected control after 7 days, (C) *O. marina* enrichment 1 after 7 days, (D) *O. marina* enrichment 2 after 7 days, (E) *O. marina* enrichment 3 after 7 days, (F) viral inoculum from seawater before enrichment on *C. tenera*, (B) *C. tenera* uninfected control after 7 days, (C) *C. tenera* enrichment 1 after 7 days, (D) *C. tenera* enrichment 2 after 7 days, (E) *C. tenera* enrichment 3 after 7 days.

Epifluorescence shows some small fluorescent particles before enrichment (Fig. 2A,F), suggesting large viruses may be present in the original viral inocula, although these likely infect a wide variety of hosts endogenous to those environments. Small fluorescent particles are observable in control cultures (Fig. 2B,G), although these measure larger than 500 nm, suggesting they may be small bacterial cells. After three passages over 7 days, some

enrichment cultures show large abundances of small fluorescent particles (Fig 2C,H), although other enrichments show little evidence of viruses. This suggests that if giant viruses are being enriched for during passaging, they are not highliy abundant in the original samples, as all enrichments for one organism began with the same viral inocula.

Removal of bacterial contamination from giant viral fractions

To remove bacteria from giant virus fractions, an antibiotic mix was added to samples. Antibiotics were chosen to target RNA polymerase, ribosomes, and cell walls; care was taken not to induce damage to membranes or DNA, as giant viruses are enveloped dsDNA viruses. Antibiotic treatment reduced the bacterial burden by ten-fold for most samples (Table 2). Bacterial cells and cellular debris are difficult to remove, and make imaging more difficult and confusing.

Table 1. Bacterial burden before and after antibiotic and lysozyme treatment of enriched virus samples.

	Pre-treatment (PFU/mL)	**Post-treatment (PFU/mL)**	**Fold change**
C. tenera uninfected control	$4x10^7$	$4x10^6$	0.1
C. tenera enrichment 1	$1x10^6$	$1x10^6$	1
C. tenera enrichment 2	$5x10^5$	$1x10^5$	0.2
C. tenera enrichment 3	$1x10^4$	$3x10^4$	3
O. marina uninfected control	$4x10^6$	$5x10^7$	12.5
O. marina enrichment 1	$1x10^8$	$1x10^7$	0.1
O. marina enrichment 2	$5x10^7$	$4x10^6$	0.08
O. marina enrichment 3	$1x10^7$	$2x10^6$	0.2

Purification of giant viral DNA

Purification of viral DNA was done from final virus enrichments to be sent for sequencing (results pending at time of this writing). Low yields were observed, although this may be indicative by good decontamination of the sample from bacteria through the use of DNase and lysozyme. It also possible that material was lost during centrifugation steps.

Table 2. DNA concentration following viral DNA purification.

	[DNA] (ng/µL)
C. tenera uninfected control	0.012
C. tenera enrichment 1	0.010
C. tenera enrichment 2	0.038
C. tenera enrichment 3	0.023
O. marina uninfected control	0.021
O. marina enrichment 1	0.018
O. marina enrichment 2	0.019
O. marina enrichment 3	0.025

Reinfection of protist hosts

After four days incubation with enriched viral fractions, hosts were imaged with FISH probes against bacteria and DAPI. The intent of this was to distinguis host nuclei, which are large and should only stain with DAPI, from bacterial cells, which are small and should hybridize with the FISH probe, from giant virus replication centers, which were expected to appear as diffuse areas of intracellular DAPI staining.

O. marina was difficult to fix, and rapidly overfixed in the presence of even low (1%) concentrations of paraformaldehyde. Only one intact cell was observed, although its morphology is not characteristic of *O. marina*, so it may be a contaminant (Fig. 3). Unfortunately, the host nucleus is not distinguishable from bacterial signal - likely a result of ingested bacteria - so future work may need to find better methods for staining nuclei.

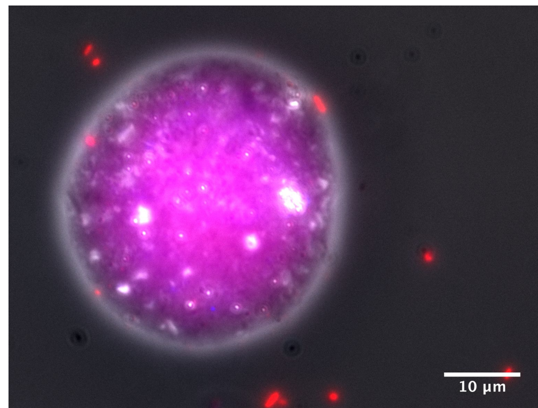

Figure 3. The only eukaryotic cell observed by FISH microscopy. Morphology suggests this is either an overfixed or stressed cell *O. marina* cell, or a contaminant. Stained with DAPI (blue) and EUB338 FISH probe (red).

Reinfection of *C. tenera* yielded variable phenotypes. Reinfection with enrichments 2 and 3 did not have any apparent phenotype (Fig. 4G,H) compared to the uninfected controls (Fig. 4A-C). However, reinfection with enrichment 1 shows a phenotype suggesting *C. tenera* is consuming more bacterial cells, as more intracellular structures are staining with bacteria-specific probe, but also may be overwhelmed by bacteria, as more bacteria are readily apparent outside the host (Fig 4D-F).

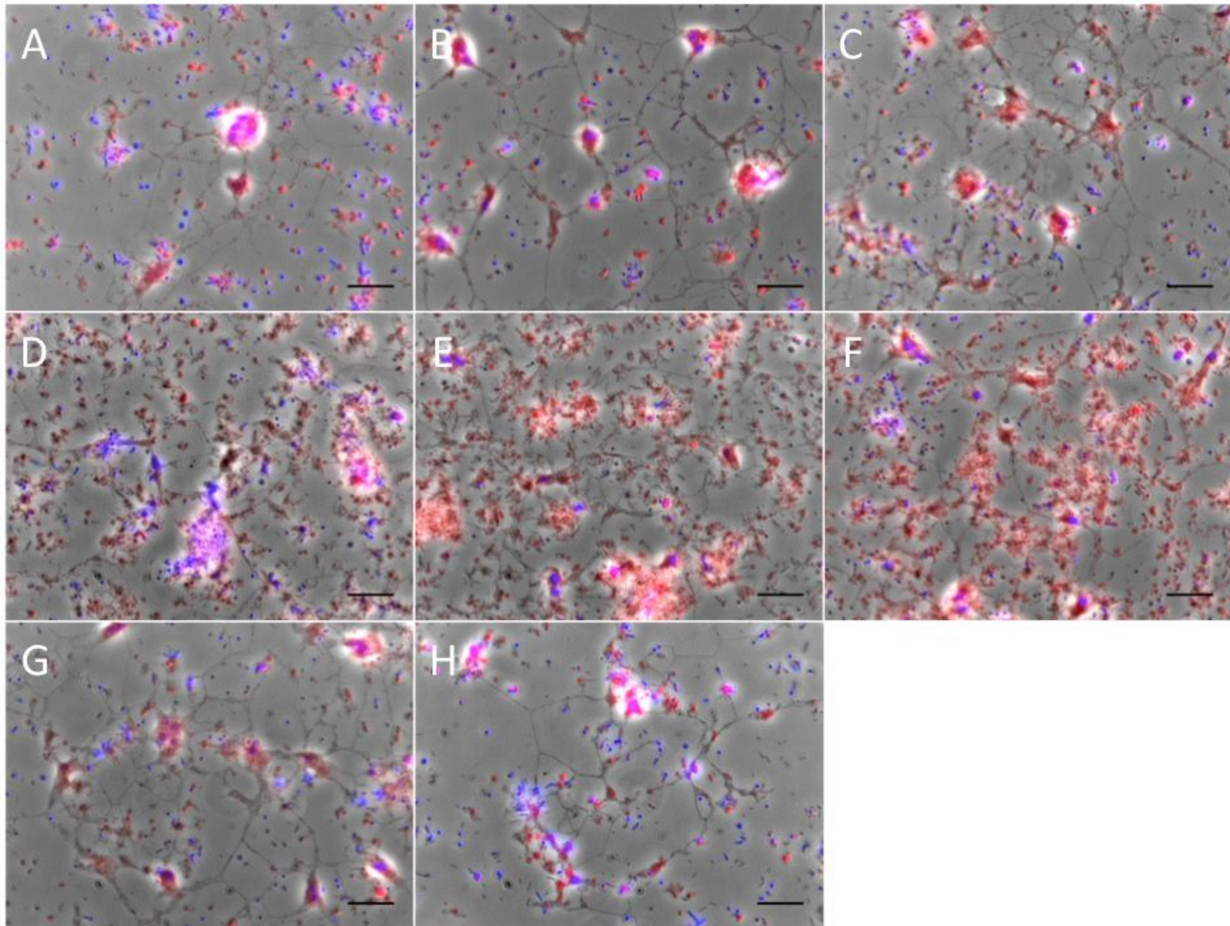

Figure 4. FISH microscopy of reinfected *C. tenera* cultures with final giant virus enrichments: (A-C) uninfected cultures, (D-F) cultures reinfected with final virus enrichment 1, (G) culture reinfected with final virus enrichment 2, (H) culture reinfected with final virus enrichment 3. Scale bar = 100 μm.

Imaging of viral fractions

TEM was employed to search for giant virions, which are known to have icosahedral morphologies, sometimes with apparent envelopes or carbohydrate capsules. This search was complicated by the fact that the hosts used are completely unexplored prior to this study, so viruses and their morphologies may also be divergent from those that are known. No clearly icosahedral particles were observed in enrichments from either *O. marina* (Fig. 5), or *C. tenera*

(Fig. 6). Many small, spherical particles were observed of various sizes, and some particles appear to have interesting structure suggestive of an envelope (Fig. 5C,G,K & Fig. 6B,C,F). Because of large amounts of bacterial contamination, it is often difficult to distinguish dead cells and debris from potential giant viruses (*e.g.* Fig 5C). Most particles observed are smaller than typical giant viruses. It is possible that large amounts of viral content were lost during final centrifugation steps. Supernatants from these centrifugation steps will be searched for giant viruses by TEM in case this has occurred.

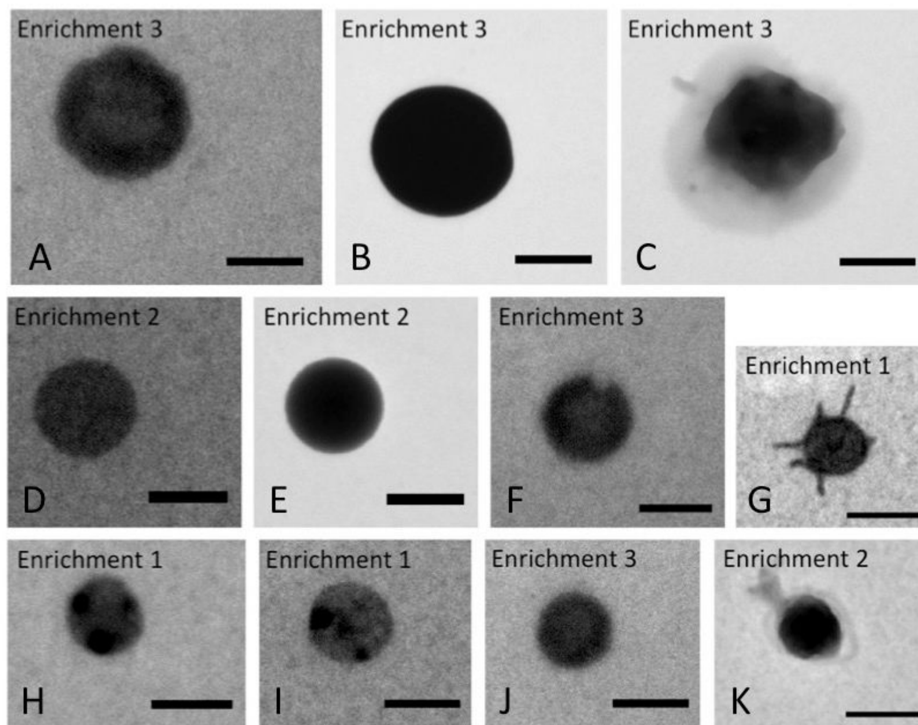

Figure 5. TEM of final giant virus enrichments from *O. marina*. Scale bar = 200 nm.

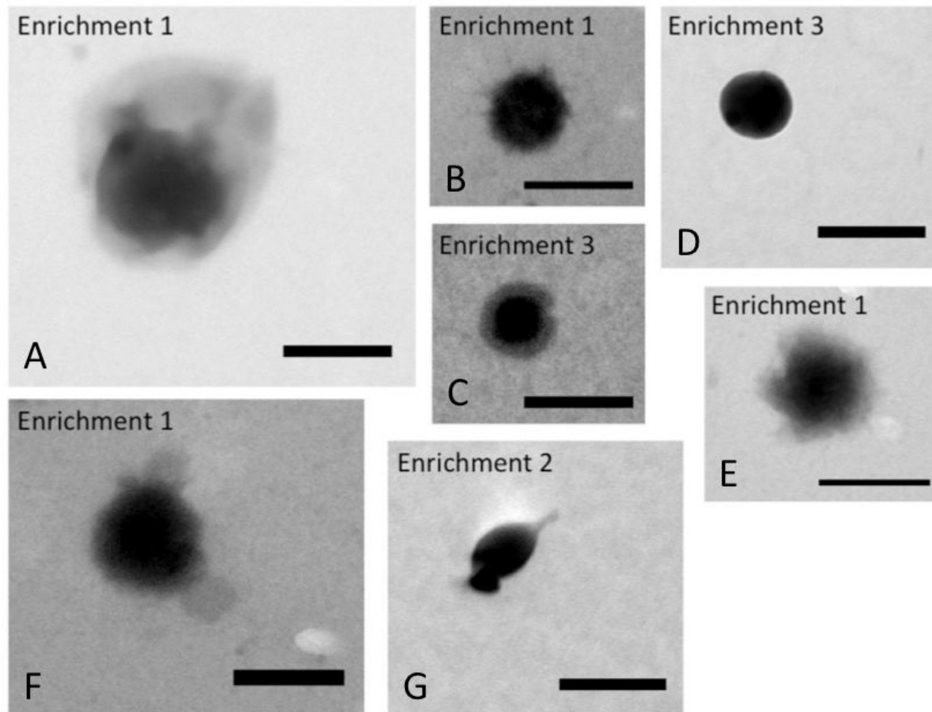

Figure 6. TEM of final giant virus enrichments from *C. tenera*. Scale bar = 200 nm.

Advice for Future Projects

The biggest hindrance to this project was the inability to separate bacterial cells from potential giant viruses. Since both hosts used are obligate predators, bacteria cannot be entirely removed from cultures. An antibiotic mix or toxin mix that does not damage membranes or DNA (since giant viruses are enveloped dsDNA viruses) needs to be devised to allow for isolation. Implementing antibacterial steps, and sufficient washing, during passaging for enrichment might greatly increase the chances of finding giant viruses, especially in TEM. Additionally, it is unclear if centrifugation was sufficient to pellet giant viruses from supernatant, so using 0.2 µm filters for concentrating and washing viruses is advisable.

In order to fully use *O. marina* as a host, better methods for fixation need to be worked out. Methods for the fixation of bacteria quickly overfixed *O. marina* to a raisin-like state, and finding *O. marina* cells is hindered by low cell abundances.

Acknowledgements

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References

1. Boughalmi, M., Saadi, H., Pagnier, I., Colson, P., Fournos, G., Raoult, D., La Scola, B. (2013) High-throughput isolation of giant viruses of the *Mimiviridae* and *Marseillesviridae* families in the Tunisian environment. *Environ Microbiol* 15(7), 2000-2007.
2. Bou Khalil, J., Andreani, J., La Scola, B. (2016) Updating strategies for isolating and discovering giant viruses. *Current Opin Microbiol* 31, 80-87.
3. Fischer, M., Allen, M., Wilson, W., Suttle, C. (2010) Giant virus with a remarkable complement of genes infects marine zooplankton. *PNAS* 107(45), 19508-19513.
4. Schvarcz, C., Steward, G. (2018) A giant virus of green algae encodes key fermentation genes. *Virol* 518, 423-433.
5. Gaelle Reteno, D., Benamar, S., Bou Khalil, J., Andreani, J., Armstrong, N., Kloe, T., Rossman, M., Colson, P., Raoult, D., La Scola, B. (2015) Faustovirus, an Asfarvirus-Related New Lineage of Giant Viruses Infecting Amoebae. *J Virol* 89(13), 6585-6594.