Tamin' the amoebae: A preliminary investigation of genetic manipulation on marine microbial predatory eukaryotes

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Introduction

Microbial eukaryotes are single-celled organisms composed of both primary producers, such as algae and diatoms, and consumers, such as flagellates and amoebae (Dawson and Fritz-Laylin, 2009). Classically, attempts to characterize and classify protists have been based upon their cell morphology and behavior (Hirst *et al*., 2011), but other than this, not much is known about the microbial eukaryotes in general. The study of these microbial eukaryotes are important because of their ubiquity, role in the natural environment, and ancestral eukaryotic features (Fritz-Laylin *et al*., 2010). Fortunately, the past decade has seen a revolution in culture-independent techniques, such as genetic studies, high-throughput sequencing, and the "omics". This has rapidly expanded the scientific understanding of microbial diversity in nature (Bass *et al*., 2009).

Marine predatory amoebae are a particular group of protists that have been understudied genetically despite their prevalence in marine habitats. One reason for this may be due to their amoeboid movement (Smirnov and Goodkov, 1999) and how most focus on the amoeba's movement to first identify it (Page 1983). Another reason may be because of the difficulty in culturing amoebae axenically (Dawson and Fritz-Laylin, 2009). Bacteria or other microorganisms are usually added to cultures as a food source, and thus pose as an impediment to contemporary studies. While some genetic information is available for free-living and especially the parasitic eukaryotes, no information is currently available for the marine eukaryotes.

The three model amoebae used throughout this study are *Corallomyxa tenera*, *Neovahlkampfia damariscottae*, and *Vahlkampfia avara*. *C. tenera* is a species that can form these large, delicate and reticulate networks. Only partial gene information is available for this organism, and this was used previously to phylogenetically place the organism (Tekle *et al*., 2007). Both *N. damariscottae* and *V. avara* are members of the class *Heterolobosea* and family *Vahlkampfiidae*, in which members move with eruptive bulges (Page and Blanton, 1985). However, not much research has been conducted in these two genera, and most studies done on the *Heterolobosea* involve species from the *Naegleria* genus. As a result, the question we wanted to answer was whether or not a genetic modification or manipulation system could be developed for organisms within these understudied groups of microbial eukaryotes. This report portrays some initial attempts at answering this question.

Methods

Amoeba strains and culture conditions. Throughout this study, *Corallomyxa tenera*, *Vahlkampfia avara*, and *Neovahlkampfia damariscottae* were used as model organisms. The media used during this experiment were either freshwater (FW) for *V. avara* or saltwater (SW) LTY for both *C. tenera* and *N. damariscottae*. For every L of FW or SW, the following components were added: 1 mL of 0.5 M MOPS buffer, 0.1 g of tryptone, and 0.1 g yeast extract. Amoeba species were grown in tissue-culture treated flasks

(Corning) aerobically at room temperature. An inverted phase contrast microscope (Leitz Diavert) was used to monitor the growth and state of each culture.

Antibiotic testing. Two antibiotic agents, hygromycin and neomycin (or G418), were tested against the amoeba strains for natural resistance. Strains were transferred to a 6-well plate containing 3 mL of media and three different concentrations of antibiotic. Initially, 10, 25 and 50 μg/mL of each antibiotic was tested, but later increased by 10x to 100, 250 and 500 μg/mL. To ensure that cells were not encysting due to nutrient limitation, media was exchanged every 24 hrs with fresh media and antibiotic. The number of live cells and cysts were counted at each time point by visualization under the Leitz microscope. Cells were counted as alive if amoeboid movement was observed.

Vector information and construction. The vectors used throughout this study are shown in Table 1 and the respective vector maps displayed in Figure 1. These *Naegleria* vectors were designed and provided by Lillian Fritz-Laylin, and were all transformed into chemically competent *E. coli* JM109 cells according to standard protocol. The plasmids were then extracted using a PureYield Plasmid Maxiprep System (Promega). Plasmid DNA was quantified using a Quantus fluorometer (Promega) and used for future experiments.

The *Corallomyxa* vector was constructed using a vector sequence, GFP gene, and the 5'-untranslated region (UTR) and 3'-UTR of *C. tenera* α -tubulin gene. The vector was amplified using corvecF (5'-GCTAGCCATTCTAGATCGAGCGCTAACCAC-3') and corvecR (5'-CTCTAGACTTAATTAAGGATCCGGCGCGCC-3'); the GFP gene was amplified using corgfpF (5'-TTTAACATCCAAAACAACTCAATTTAAAACATGGCTAGCAAAGGA GAAGAACTTTTCACT-3') and corgfpR (5'-AGAAGAGAATAACAAAAAAAAAAGTGATGAGATTT ATTTGTAGAGCTCATCCA TGCCATG-3'); and the 5'-UTR and 3'-UTR were amplified using atb5F (5'-GGCGCGCCGGATC CTTAATTAAGTCTAGAGCTTTTGCAGGTTCTCTCAAGGGTTTTGAG -3'), atb5R (5'-AGTGAAAAG TTCTTCTCCTTTGCTAGCCATGTTTTAAATTGAGTTGTTTTGGATGTTAAA-3'), atb3F (5'-CATGG CATGGATGAGCTCTACAAATAAATCTCATCACTTTTTTTTTTGTTATTCTCTTCT-3'), and atb3R (5'-GTGGTTAGCGCTCGATCTAGAATGGCTAGCATCTTTTAAAATTCTTCGCCTTCGCCACCT-3'). Amplification was done using the Phusion High-Fidelity PCR kit following the manufacturer's protocol (New England BioLabs). Amplification products were subsequently gel-extracted and quantified using the Quantus fluorometer. Then equal molecular ratios each product was ligated via isothermal cloning. Ligation was performed for approximately 1.5 hrs before transforming *E. coli* JM109 (chemically competent) and *E. coli* DH5α (electrocompetent). Cells were then shaken in LB broth to recover for 1 hr before spreading on LB + ampicillin 100 μg/mL plates at 37°C overnight. Colonies were picked into LB broth with 100 μg/mL ampicillin, and grown at 37°C. The plasmid was checked to

Table 1.

Plasmid #	Full name	Antibiotic resistance
1	pNgr_histoneH3_mCh	Hygromycin
2	pNGr_homorecombo_GAPDH_GFP_neo	Neomycin
3	pNgr_transient_GFP	Neomycin
4	pNGr_homorecombo_GAPDH_GFP_hygro	Hygromycin

ensure that the ligation procedure worked by doing a colony PCR using atb5F and atb3R primers.

Transfection procedure. Lipofectamine 2000 and 3000 (Invitrogen) were both tried as transfection reagents according to the manufacturer's protocol. Briefly, 14 and 5 µg of plasmid DNA were added to diluted Lipofectamine 2000 and 3000 reagents respectively. The mixture was then incubated at room temperature for 5 mins to create the DNA-lipid complex, which was subsequently added to each culture. Strains were grown with the DNA-lipid complex for various days, at which point the media was aspirated out and replaced with fresh media containing the appropriate antibiotic at finally 100 µg/mL. All plasmids were combined with each reagent and added to cell cultures. Cell numbers were counted

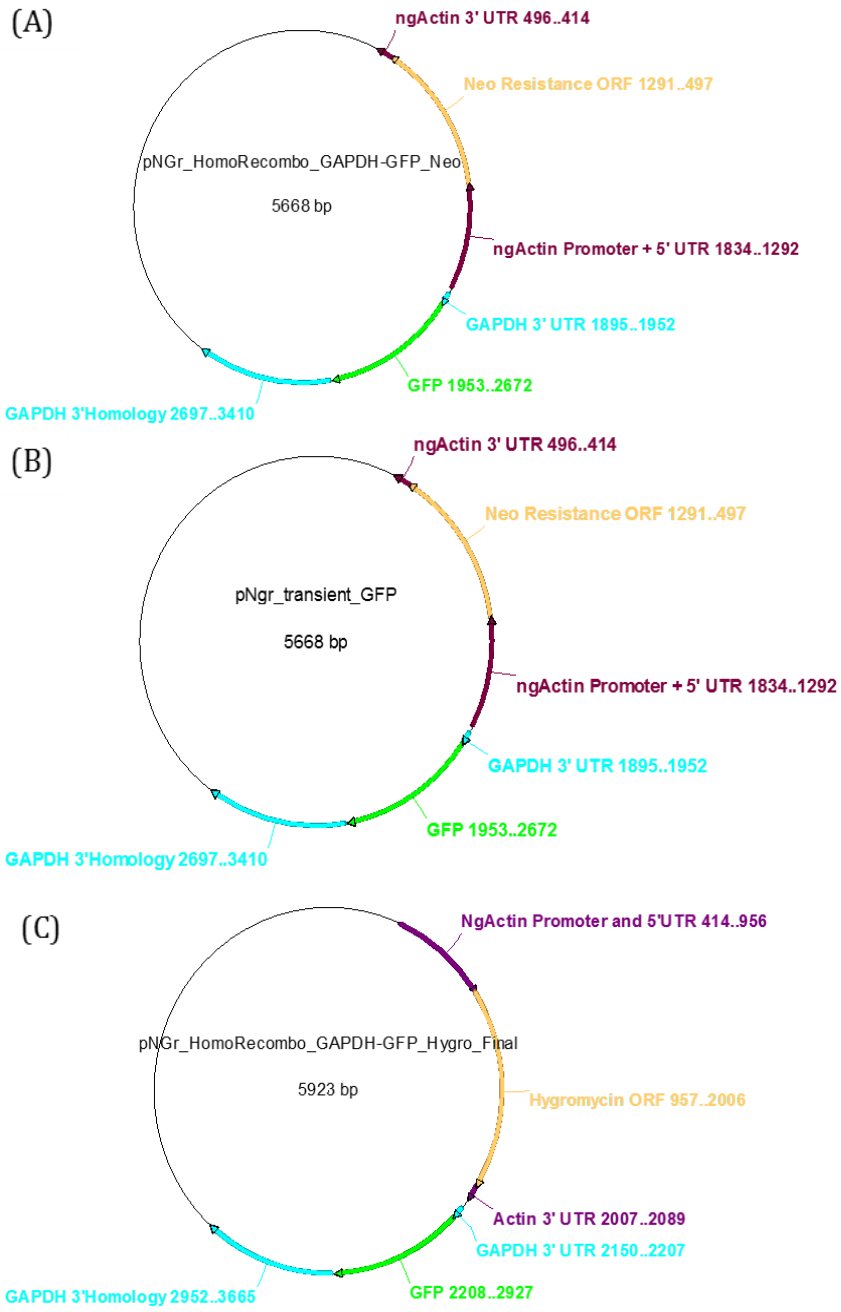

Figure 1. Vector maps of plasmids used for transfection and transformation. Three of the four vectors used throughout this study are shown. Vector map of plasmid #1 is unavailable at this time. (A and B) Plasmid #2 and #3, respectively, with *Naegleria* alpha-tubulin promoter gene and the 5'- and 3'-untranslated region (UTR) flanking a neomycin resistance gene. (C) Plasmid #4 with *Naegleria*, constructed the same way as plasmids #2 and #3 except with a hygromycin resistance gene instead.

manually to assess whether the transfection procedure worked.

Transformation procedure. For transfection, plasmid #3 (pNgr_transient_GFP) was used. *V. avara* were grown in 225 cm^2 tissue-culture treated flasks for 3 days. The spent medium was then decanted, and the flask washed gently once to remove as much bacterial cells as possible. Amoebae cells were removed from the bottom of the flask using a cell scraper, and then pelleted at 10,000 x g for 10 minutes. Cells were resuspended in phosphate-buffered saline (PBS) solution and transferred to chilled 0.4 cm electroporation cuvettes containing approximately 50 µg of plasmid DNA. Electroporation was done using a Bulldog NEPA GENE electroporating system using varying poring pulse voltages: 350V, 500V and 850V. All other parameters were kept constant during electroporation. After electroporation, cells were transferred to a 6-well plate containing fresh media and

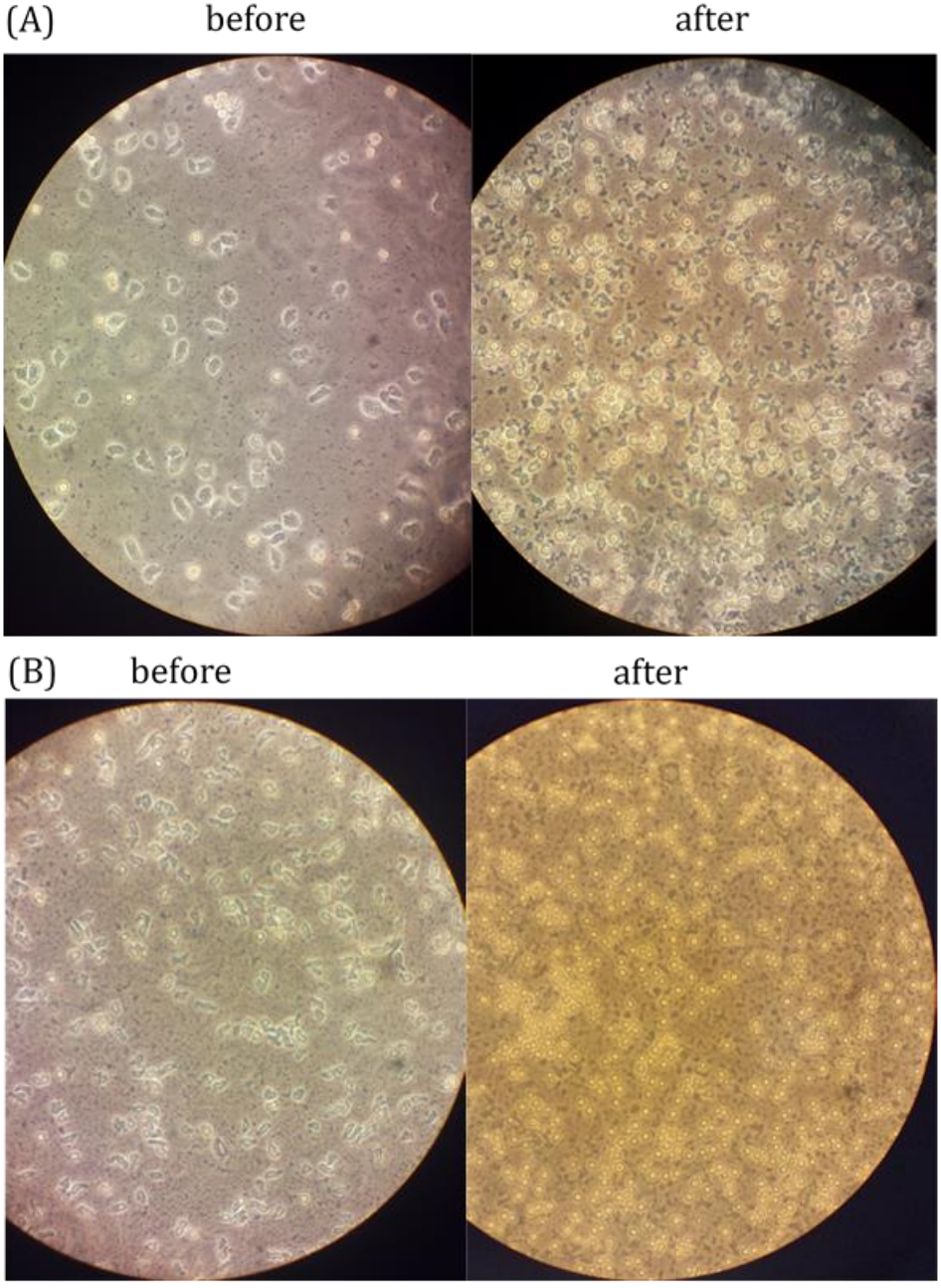

Figure 2. Effect of hygromycin and neomycin on cell growth. Both *V. avara* and *N. damariscottae* were tested for their sensitivity to hygromycin and neomycin. (A) *V. avara* cells before and 2 days after addition of 100 µg/mL of hygromycin. The cels that are not in cyst form appear to have membranes that look perforated, compared to before antibiotic addition where the cell's boundary appears smooth. Cells exposed to neomycin appeared similar after 2 days of incubation. (B) *N. damariscottae* before and 2 days after addition of 100 µg/mL hygromycin. Both hygromycin and neomycin addition prompted all the cells to form cysts. Photos were taken with a Samsung Galaxy S4 smartphone and with a microscope eyepiece-cellphone adapter, 3D printed by Sebastian Kopf.

incubated at room temperature aerobically. The media was replaced after 24 hours with fresh media and either 100 or 500 µg/mL of neomycin. Cells were observed under the microscope for cell counts before and after addition of antibiotic.

Results

Antibiotic tests. Antibiotic testing was done on *V. avara* and *N. damariscottae* only, and not on *C. tenera*. After 1 day of incubation in 10, 25 or 50 µg/mL of either hygromycin or neomycin, live cell counts were above 300 in every field observed. Media was aspirated out and replaced with fresh media containing 100, 250 or 500 µg/mL of antibiotic. After two days, *V. avara* cells were either in cyst forms or appeared sick (Figure 2A). The *N. damariscottae* cultures, on the other hand, were completely full of cysts (Figure 2B). Table 2 summarizes the live cell and cyst counts with each final antibiotic condition.

Table 2.

V. avara	Hygromycin 100	Hygromycin 250	Hygromycin 500	Neomycin 100	Neomycin 250	Neomycin 500
day 0	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC
day 2	0.018701165	0.03870743	0.035697926	0.869725132	0.626778571	0.154008699
N. damariscottae	Hygromycin 100	Hygromycin 250	Hygromycin 500	Neomycin 100	Neomycin 250	Neomycin 500
day 0	10.98723645	11.70959417	10.76143559	TMTC	TMTC	TMTC
day 2	TMTC*	TMTC*	TMTC*	TMTC*	TMTC*	TMTC*

TMTC = too many to count for live cells; TMTC* = too many to count for cysts

Transfection using Lipofectamine. Lipofectamine 2000 and 3000 reagents were used to transfect the 4 *Naegleria* vectors into *V. avara*. Table 3 gives the live cell to cyst ratios with total numbers with the Lipofectamine experiment before and after antibiotic addition. Overall, the ratios seemed to decrease after antibiotic addition, whereas total cell counts remained similar. The exception was when pNGr_homorecombo_GAPDH-GFP_neo (plasmid #2) was used because there were overall less cells after antibiotic treatment.

Electroporation of *V. avara*. We attempted transformation of *V. avara* using pNgr_transient_GFP (plasmid #3) using the Bulldog NEPA GENE electroporator. Three different poring pulses were attempted: 350V, 500V and 850V. When the electroporated cells were inoculated back into fresh media, live cells were observed, usually less than 10 per field of view. The cultures after 350V had the highest number of remaining cells, whereas 500V and 850V live cell counts were comparable. After adding 100 µg/mL neomycin and fresh media, the number of viable cells increased after one day (Figure 3), in some cases more than double the cell count from before neomycin addition.

Table 3.

Plasmid-Lipofectamine	day 1		day 2	
	live/cyst	total	live/cyst	total
#1-3000	1.2	148.5	0.22069	177
#1-2000	0.68182	175.75	0.15987	177.75
#2-3000	0.55082	236.5	0.3444	162
#2-2000	0.68024	206.25	0.20825	153.75
#3-3000	2.29032	102	1.14851	108.5
#3-2000	2	96.75	1	101
#4-3000	2.06944	110.5	1.22018	121
#4-2000	1.67883	91.75	1.57923	118

Vector construction. Vector construction specific for *Corallomyxa tenera* was attempted in this study. PCR

amplification using the 4 primer pairs separately resulted in bands of the expected sizes (atb5F/R: 145bp; atb3F/R: 161bp; corGFP-F/R: 740bp; and corVecF/R: 4800bp) for a total vector size of approximately 5846 bp. Unfortunately, the ligation procedure through isothermal cloning did not work (Figure 4) and that the plasmid constructed would not work for our future transformation experiments on *Corallomyxa tenera*.

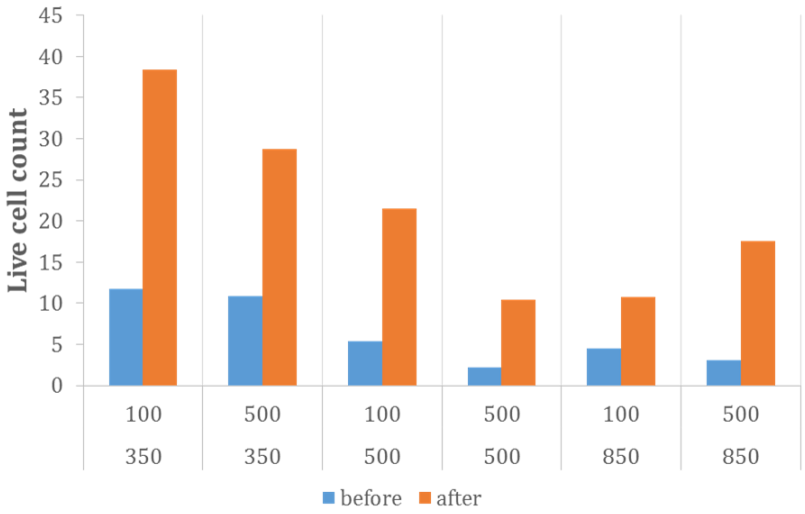

Figure 3. Preliminary results on *V. avara* electroporation. Cells were electroporated at different voltages (bottom number on x-axis), and subsequently exposed to different µg/mL of neomycin (top number on x-axis). Within one day, the number of live cells increased for every condition shown. The blue bars represent cell numbers before antibiotic addition and orange bars one day after adding neomycin.

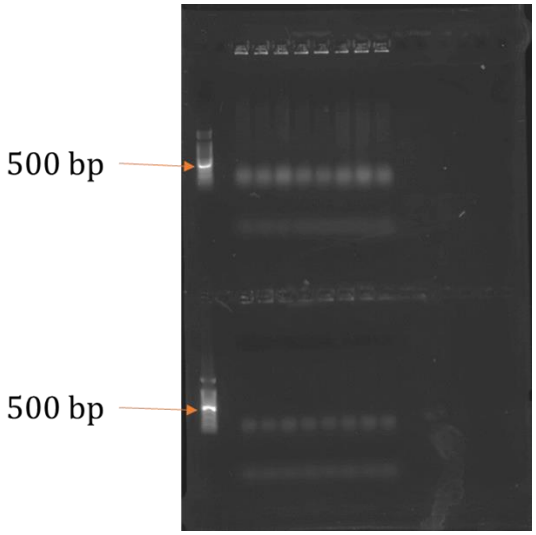

Figure 4. Gel image to check for ligated *Corallomyxa* vector. The vector was checked to make sure the 5'-UTR, GFP, and 3'-UTR genes were ligated together properly. Colony PCR was done on transformed *E. coli* JM109 and DH5α using atb5F and atb3R primers that would produce an expected band size of approximately 1000bp. The top of the gel were PCR amplifications from JM109, and the bottom of the gel from DH5α. As indicated, the brightest band of the ladder is 500bp long. All of the bands we obtained from PCR was around 300bp long, which would be the approximate size of the 5'-UTR and 3'-UTR genes only. Unfortunately, this indicates that our ligation did not work and so do not have a vector for transforming *Corallomyxa*.

Discussion and conclusions

Both hygromycin and neomycin are aminoglycoside antibiotics and in general inhibit protein synthesis in eukaryotes. Hygromycin specifically seems to promote misreading during translation, as well as inhibits ribosomal translocation by retaining the tRNA in the acceptor site of the ribosome, preventing elongation of the peptide chain (Cabanas *et al*., 1978). Neomycin can cause multiple effects in the eukaryal cell, including miscoding and inhibition of ribosomal translocation, peptide release, and ribosome recycling (Borovinskaya *et al*., 2008). Seeing as how both antibiotics seem to exert multiple effects on protein synthesis in previous studies, it was curious to see that our initial antibiotic tests did not work. In fact, within the first 24 hours after adding either 10, 25, or 50 µg/mL, both *V. avara* and *N. damariscottae* more than doubled in live, motile cells,

making counting impossible without introducing a large human error. From these results, we decided to increase the concentrations 10-fold because the primary goal was to find a concentration in which the cells died. After 2 days at 100 μg/mL, we observed an effect on the cell counts and viability for both organisms. However, *N. damariscottae* seemed to just go into cyst form upon antibiotic addition instead of dying like the *V. avara* cells. Thus, further antibiotic testing needs to be done on *N. damariscottae*, specifically with concentrations between 50 and 100 μg/mL of hygromycin or neomycin. Due to this result, only *V. avara* was used throughout the rest of the experiments.

With *V. avara* as our model organism, we tried genetic modification using two different techniques: transfection via Lipofectamine reagent and direct transformation by electroporation. The two different Lipofectamine reagents (2000 and 3000) were combined with the 4 different vectors designed originally for transforming *Naegleria*. We used these vectors because *Naegleria* is a close relative of *Vahlkampfia*, therefore performing this pilot experiment would indicate whether the procedure would work at all or not. Unfortunately, the two pNGr_homorecombo vectors (plasmid #2 and #4) were mixed up, and so the cultures were exposed to the wrong antibiotic. However, this mix-up could explain the results shown in Table 3. In all combinations of Lipofectamine and vector, the ratio of live cells to cysts decreased, which is expected knowing that the antibiotic was added. However, for plasmid #2, we observed a decrease in total cell count that was not seen with any of the other plasmids. With plasmid #4, hygromycin was added but for some reason did not seem to have any effect on total cell number compared to when neomycin was added to cultures transfected with a hygromycin vector. From these results, it was not clear whether there were cells transfected with either pNGr_homorecombo vectors. Regardless, we found with the other two vectors (plasmid #1 and #3) that the total number of cells stayed constant but the number of live cells decreased. Yet, there were still live cells, indicating that maybe the transfection procedure worked.

Similar results were detected with the electroporation experiment. The first concern was whether the cells would survive the electroporation procedure, thus attempted transformation at 3 different pulsing voltages: low at 350V, medium at 500V and high at 850V. Immediately after electroporation, some live cells were observed across all voltages, with the 350V having the highest number of live cells at the start. After adding neomycin, cell numbers more than doubled with all the cultures, hinting that maybe these cells might be transformed with the vector. This was very promising, as it may indicate that transformation of predatory marine amoebae is possible.

Lastly, we attempted to construct a vector to transform *Corallomyxa tenera* with, as we were very interested in this organism, particularly in its ability to form these structures that mimic neural networks (Figure 5). Because of this peculiar phenotype, we were hoping to develop a genetic system to study this organism. The individual components of the vector were amplified separately, and then ligated together. However, when a PCR amplification of the 5'-UTR, 3'-UTR, and GFP genes was done, the resulting band corresponded to only the 5'-and 3'-UTR genes. In other words, the ligation was not successful, and hence a vector to use for *C. tenera* transformation was not constructed.

In conclusion, the overall project was very much preliminary. If given more time, each experiment would be repeated to ensure that the results are reproducible. A lot of future experiments could be done, given the results shown here. Regardless, the experiments performed here show potential and promise – these microbial eukaryotes

might be genetically tractable, which would be critical in the continued study of these organisms.

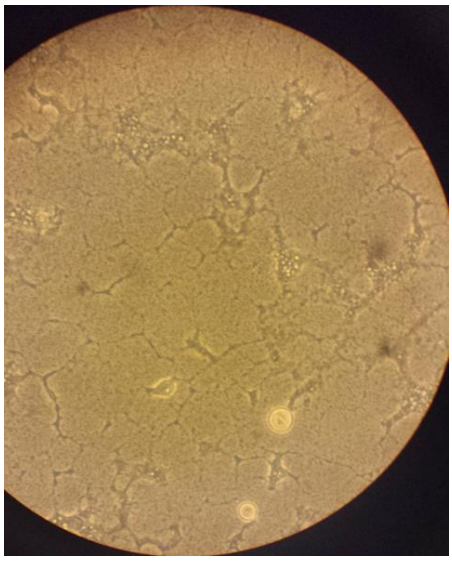

Figure 5. Image of *Corallomyxa tenera*. A microscope image of *C. tenera* was taken through the eyepiece. The elongated structures look like complex neural networks, and is the typical phenotype of this organism. The phase-bright round structures seen are fruiting bodies, though what they are exactly is currently unknown.

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References

1. Dawson SC, Fritz-Laylin LK. 2009. Sequencing free-living protists: the case for metagenomics. *Environ. Microbiol.* 11: 1627-1631.
2. Hirst MB, Kita KN, Dawson SC. 2011. Uncultivated microbial eukaryotic diversity: a method to link ssu rRNA gene sequences with morphology. *PLoS ONE*. 6: e28158.
3. Fritz-Laylin LK, Prochnik SE, Ginger ML, Dacks JB, Carpenter ML, Field MC, Kuo A, Paredez A, Chapan J, Pham J, Shu S, Neupane R, Cipriano M, Mancuso J, Tu H, Salamov A, Lindquist E, Shapiro H, Lucas S, Grigoriev IV, Cande WZ, Fulton C, Rokhsar DS, Dawson SC. 2010. The genome of *Naegleria gruberi* illuminates early eukaryotic versatility. *Cell*. 140: 631-642.
4. Bass D, Howe AT, Mylnikov AP, Vickerman K, Chao EE, Smallbone JE, Snell J, Cabral Jr. C, Cavalier-Smith T. 2009. Phylogeny and classification of Cercomonadida (Protozoa, Cercozoa): *Cercomonas*, *Eocercomonas*, *Paracercomonas*, and *Cavernomonas* gen. nov. *Protist*. 160: 483-521.
5. Smirnov AV, Goodkov AV. 1999. An illustrated list of basic morphotypes of Gymnamoebia (Rhizopoda, Lobosea). *Protistology*. 1: 20-29.
6. Page FC. 1983. Marine Gymnamoebae. Institute of Terrestrial Ecology Natural Environment Research Council. Lavenham, Suffolk: Cambridge, UK.
7. Tekle YI, Grant J, Cole JC, Nerad TA, Anderson OR, Patterson DJ, Katz LA. 2007. A multigene analysis of *Corallomyxa tenera* sp. nov. suggests its membership in a clade that includes *Gromia*, Haplosporidia and Foraminifera. *Protist*. 158: 457-472.
8. Page FC, Blanton RL. 1985. The Heterolobosea (Sarcodina: Rhizopoda), a new class uniting the Schizopyrenida and the Acrasidae (Acrasida). *Protistologica*. 21: 121-

132.
9. Cabanas MJ, Vasquez D, Modolell J. 1978. Dual interference of Hygromycin B with ribosomal translocation and with aminoacyl-tRNA recognition. *Eur. J. Biochem.* 87: 21-27.
10. Borovinskaya MA, Shoji S, Fredrick K, Cate JHD. 2008. Structural basis for hygromycin B inhibition of protein synthesis. *RNA*. 14: 1590-1599.