Title
Toward the genetic engineering of disease resistance in oysters

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Final Report: Toward the genetic engineering of disease resistance in oysters
SeaGrant R/A -112
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The focus is on research conducted and the progress made toward the achievement of all specific aims outlined in the original proposal. Three abstracts describing aspects of our work in oysters were presented at national and international meetings.

Specific Aim: Test methods of introducing pantropic vectors into *C. virginica* embryos to create transgenic oysters

Approaches to introduce the vectors into oyster embryos included the following:
1) incubation of sperm with pantropic vector (LLRNL: expresses luciferase reporter gene from the MoMLV LTR; see attached manuscript for further details)
2) Electroporation of oyster embryos followed by incubation with vector
3) Microinjection of vector into embryo or into perivitelline space
4) Chemical treatment to disrupt chorion followed by incubation with pantropic vector

Introduction of transgene using retroviral infection

In order to assess if pre-incubation of gametes with the retroviral vector encoding a luciferase reporter gene is feasible we conducted the following studies. Gametes were aspirated from reproductively mature oysters and the sex was determined using phase contrast microscopy. Sperm were placed into 1.5 ml tubes on ice. Ova were rinsed with fresh seawater and held at 15 EC for 45 min prior to fertilization. Sperm was incubated with vector at a MOI of 2 for 15 minutes at 15 EC. Sperm were diluted with 20 ppt seawater (total volume of 100μl), added 100 μl of LLRNL vector to achieve 14 ppt (435 mOsm). Control sperm were incubated in sea water at 435 mOsm as above. Fertilization was conducted in 500 ml beakers as follows:

Trial I (Two females (F) and one male (M) were individually crossed in this trial)

#1F x #20M	#1F x #20M	#9F x #20M	#9F x#20M
MOI of 2 with vector	Control	MOI of 2 with vector	Control
60,000 eggs	60,000 eggs	60,000 eggs	60,000 eggs
1 egg: 100 sperm	1 egg: 100 sperm	1 egg: 100 sperm	1 egg: 100 sperm

Male and female gametes were mixed and incubated for 30 min for fertilization. Subsequently, fertilized eggs were decanted at 10 eggs/ml into 8 L, aerated oyster bags. Upon hatching at 24 h, larvae were fed *Isochrisis galbana* (T-Iso) daily. Larvae were enumerated and assessed for luciferase activity (see below) 3 d after fertilization.

Treatment	# eggs stocked	# veligers
#1F with vector	60,000	50
#1F control	60,000	10
#9F with vector	60,000	285
#9F control	60,000	3

Trial II

Gametes were collected and handled as in Trial I with the following exception: Ova were held for 1 hr 30 minutes prior to fertilization. As an additional control aliquots of sperm were also incubated with Bald LLRNL: 75μl sperm + 75 μl bald vector for 10 min.

In order to assess the ability of ATA, shown to degrade the chorion of shrimp ova, to aid in the efficacy of retroviral vector infection, ova were incubated with 1 mM ATA for 10 min. Vector was then added to the egg solution (75 μl of LLRNL vector) and incubated for 10 min. Eggs were then fertilized: 38 μl of sperm solution was added to the eggs. All eggs were fertilized simultaneously and maintained and sampled as above.

Summary of Treatments:

Female x Male	Treatment	# eggs	egg: sperm ratio
#3F x #27M	LLRNL Vector	53,000	1:113
#18F x #27M	LLRNL Vector	53,000	1:113
#3F x #27M	LLRNL Bald	53,000	1:113
#18F x #27M	LLRNL Bald	53,000	1:113
#3F x #27M	ATA w/ vector	53,000	1:113
#18F x #27M	ATA w/vector	53,000	1:113

After 3 d of culture the following number of veligers were enumerated:

Treatment	# eggs stocked	# veligers
#3F with vector	53,000	680
#3F bald	53,000	340
#3F ATA w/ vector	53,000	0
#18 w/ vector	53,000	1760
#18 bald	53,000	180
#18 ATA w/ vector	53,000	0

Embryos resulting from the above treatments were be lysed at 3–5 days post-infection and the cell lysates tested for luciferase activity.

Trial 1 results which indicate that prefertilization of sperm with the retroviral vector were ineffective are illustrated below:

SampleBlank (cpm)		Sample (cpm)	Total cpm (sample- blank)
#1F Control	11353	12250	897
#1F Vector	11353	11984	631
#9F Control	11353	12038	1535
#9F Vector	11353	12888	685

Trial II mirrors Trial 1 above as shown by the luciferase counts below. The low survival of oyster larvae as compared to initial trials suggests that the condition of the oysters in laboratory may have compromised the competence of the gametes (by being held under laboratory conditions for several weeks).

SampleBlank (cpm)	Sample (cpm)	(sample-blank).	
#3F with vector	15455	15302	-153
#3F bald	15455	18256	2801
#18 w/ vector	15455	13808	-1647
Same # veligers as bald			
#18 w/ vector	15455	14456	-999
(1580 veligers)			
#18 bald	15455	13652	-1803

Thus, retroviral infection of oyster gametes prior to fertilization does not appear to be an efficient means of introducing transgenes into oyster embryos. Several investigators have successfully used Dr. Burns vector to produce transgenic animals via microinjection. However, given the small size of fertilized oyster embryos in relation to *Danio* embryos into which transgenes were successfully introduced, we realized that an alternative method was required. Another successful production of transgenic *Danio* fish was conducted using the mariner plasmid (Raz et al. 1997) via microinjection of fertilized embryos. In addition, via collaboration with the Boulo lab in France, we realized that electroporation of oyster cells resulted in excessive cell death and was an inefficient method of transgene introduction (Boulo et al. 2000). We also realized that the LLRNL vector would not be effective and that we needed to use the mariner plasmid and either lipofection or, possibly particle bombardment. We needed to first create a reporter plasmid in which the *C gigas* actin promoter drives the luciferase (reporter) or selected transgene (i.e., tachyplesin) to assess the efficacy of this promoter to drive the selected transgene. As such the Burns lab developed such a plasmid (GiaLuc) for use in lipofection studies. In addition, with a final goal of using the mariner plasmid in combination with and mRNA transposase for stable integration of the plasmid DNA into the host cell genome, we needed to assess the efficacy to lipofecting mRNA.

Specific Aim: Optimize transfection protocols to transfer transgenes into oyster heart cells

Plasmids and mRNA

The LLRNL vector, GiaLuc plasmid and luciferase mRNA were produced by the Burns lab at UC San Diego. The GL3Basic plasmid (Promega) was used as a negative control. The pLuc plasmid was provided by the Kurath lab at the Western Fisheries Research Center (Seattle, WA) and was used as a positive control for luciferase transfection.

Oysters cultures

Adult cultured *C. virginica* oysters were obtained from various sources in California, Washington, Alabama, and Maine and were held up to several weeks in a 15 °C, 600 L recirculating system with weekly seawater changes, and fed algal paste (Reed Mariculture, San Jose CA). When *C. virginica* was unavailable or upon examination of the integrity of our vectors, we examined *C. gigas* as an alternate model species. Pacific oysters were obtained from Washington state or California and maintained as above.

Heart tissue primary cultures

Enzymatic dissociation of cardiac tissue followed the procedure of Boulo et al. 1996. During 2002-2003 adult Pacific oysters (*C. gigas*) were obtained by the Pacific Shellfish Institute from various locations around Puget Sound, Washington. The oysters were held for several weeks in non-recirculating tanks at 15°C with weekly water changes and daily feeding of algal paste (Reed Mariculture, San Jose CA). Oysters were placed on fresh seawater for 2 hours before dissections. Oysters were shucked and the ventricle and auricles were excised and placed on ice in a modified HBSS. Hearts from 7-20 individuals were pooled, minced with sterile scalpels to 1 mm^3 and placed in a beaker containing 100 mL of 0.012 % pronase in mHBSS with 200 U/ml penicillin and 200 μg/ml streptomycin. The minced tissue was digested at 4 °C for 14-16 hours with slow stirring and then poured through a 150 μm mesh, transferred to 50 mL tubes and rinsed three times with mHBSS by centrifugation (800 g, 5 min, 4 °C). Cells were resuspended in media (below), assessed for viability by trypan blue exclusion (0.2%) and adjusted to 1 x 10^7 viable cells/mL. The cell suspension was then plated into 24-well plate wells (200 μL, 2 x 10^6 cells).

Transfections

Transfections were conducted 1 h after plating cells. Lipofectamine 2000 (Gibco) was added to 50 μL Opti-MEM I medium (Gibco) an incubated 5 min, followed by addition of varying amounts of vector or GiALuc plasmid in an additional 50 μL Opti-Mem I medium. The mixture was incubated 20 min at room temperature and added to heart cultures in 500 μL medium without antibiotics. After 6 h the transfection medium was replaced with standard medium.

In order to optimize the introduction of transgenes, we assessed the concentration of Lipofectamine 2000, plasmid concentration, cell density and media used in the lipofection assays. The GiaLuc vector was applied at the time of plating at concentrations of 0.4 and 0.97 μg DNA per well, cell density varied between 1-4 x 10^6 cfu/well, Lipofectamine concentrations assessed included 1.25, 2.50 and 5.00 μl per well.

Reporter gene assays with C. virginica

Reporter gene assays were conducted 72 h after LLRNL or Geo4.8 vector infection and 48 h after transfection with GiALuc plasmid. Luciferase expression was detected using a "Luciferase Assay System with Reporter Lysis Buffer" (Promega) using a modified version of the protocol for detection with a scintillation counter. The modifications include an increase in sample volume and additional steps which result in the harvest of unattached cells as well as attached cells. Well contents (media and unattached cells) were transferred to a 0.5 mL centrifuge tube and centrifuged (347 g, 5 min, 4 °C), washed once in mHBSS and the supernatant removed. At the same time 50 μL of 1x lysis reagent was added to the emptied wells, contents were scraped and the suspension was transferred to the tube containing the pellet of cells that were in suspension. The contents were pipetted ten times to lyse all cells and the tube was centrifuged (1800 g, 5 min, 4 °C) to pellet nuclei. A sample of the supernatant (7 μL) was taken to determine protein concentration (BioRad DC protein assay) and 35 μL was added to 175 μL assay reagent in a 0.5 mL clear centrifuge tube, which was placed in a plastic 7 mL scintillation vial. The vial was placed in the scintillation counter (Beckman LS 5000 TD) and light output (cpm) was measured after 1 min. The scintillation counter was set to single photon mode with 0.5 min single photon time and 0.5 min single photon interval.

As illustrated in Figures 1 and 2 below, 5.0 μl of Lipofectamine appeared most efficient. In addition, we discovered that the KS medium was superior to the JM-7 medium using 1.0 μg vector DNA and 2 x 10^6 cells per well. As a result of these trials, our standard protocol employed 2 x 10^6 cells, 5.0 μl Lipofectamine, and 1.0 μg DNA in the KS medium.

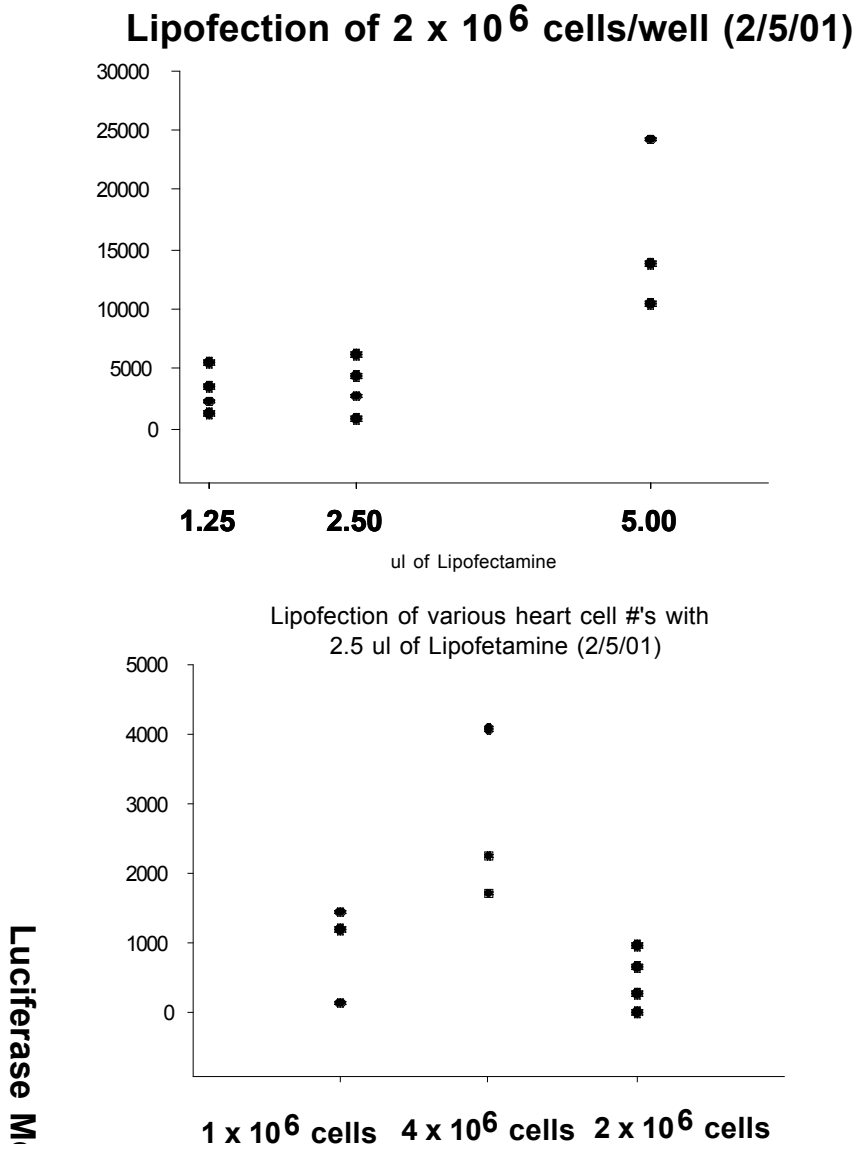


Figure 1. Optimization of lipofection with *C. virginica* primary heart cultures using varying concentrations of Lipofectamine 2000 (top graph) and two media based on published data (KS media) and a medium we created based on research we conducted in a related project funded by California Sea Grant in which we examined the key components of oyster hemolymph. This medium attempted to recreate the major components of oyster hemolymph.

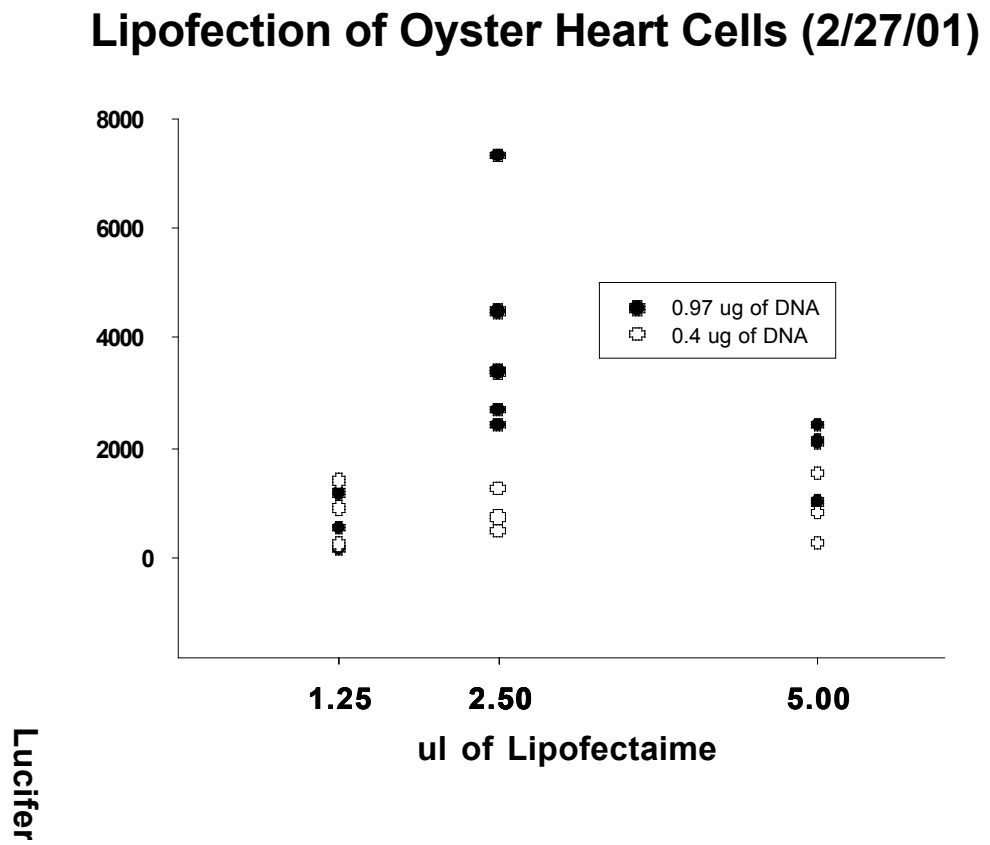


Figure 2. Further optimization of introducing a reporter transgene via lipofection using varying amounts of Lipofectamine 2000 and plasmid DNA.

Reporter gene assays with C. gigas

Reporter gene assays were conducted 48 h after transfection with GiaLuc plasmid. Luciferase expression was detected using a Luciferase Assay System with Reporter Lysis Buffer" (Promega) using a modified version of the protocol for detection with a luminometer. Well contents (media and unattached cells) were transferred to a 1.5 mL centrifuge tube and centrifuged (650 g, 5 min, 4°C), washed once in mHBSS, and the supernatant removed. At the same time, 50 μL of 1x lysis reagent was added to the emptied wells, contents were scraped and the suspension was transferred to the tube containing the pellet of cells that were previously in suspension. The contents were pipetted ten times to lyse all cells and the tube was centrifuged (2000 g, 5 min, 4°C) to pellet nuclei. A sample of the supernatant (35 μL) was added to 175 μL of assay reagent in a 1.5 mL centrifuge. Luminescence was measured using a Reporter Microplate Luminometer (Turner BioSystems, Sunnyvale CA). With the extra sensitivity option on, the relative light units were measured following the manufacturers instructions.

Optimization of tranfection in different media for plasmid and mRNA introduction into oyster cardiac cells

Given the use of different vectors (GiaLuc, pLuc and luciferase mRNA) for the introduction of transgenes into oyster cells, we re-examined the best medium for the lipofection assays. The initial medium (KS) used in these studies was a modification of that reported by Kleinschuster and Swink (1993) for culture of *Perkinsus marinus*. Components are insect tissue culture tested reagents from Sigma Chemical Company unless otherwise noted. L-15 medium (Sigma) was adjusted to pH 7.4 and to 750 mOsm with the addition f 14.9 g/L sea salts. This basal medium was supplemented with 0.005% taurine, 0.05% glucose, 0.03% galactose, 0.05% fructose, 0.05% trehalose, 0.1% yeast extract (Difco), 0.3% lactalbumin hydrosylate, 1x MEM vitamins, 1x lipid mix, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2% fetal bovine serum (FBS, Gibco).

After several attempts to achieve a culture of healthy, well-attached oyster heart cells, a simpler medium (2X L-15, 2% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin) was tested along side the KS medium to evaluate the transfection efficacy of these two media. Figure 3 illustrates that the 2X L-15 was far superior to the KS medium for the introduction of luciferase mRNA into oyster cells. That the GiaLuc was not successfully introduced into *C. gigas* cardiac cells, the species from which the promoter was derived, was perplexing. However, the ease of introduction of mRNA was an exciting finding as this suggests that we can introduce the transposase mRNA to further examine the efficacy of our vectors before the use of particle bombardment is examined, an expensive task.

In order to further examine why the GiaLuc plasmid was no longer effective, we repeated the study using a more sensitive luminometer as shown by Figure 4. Although a small signal was observed in the 2X L-15 medium only with the GiaLuc, the efficiency was negligible as compared to previous experiments (Figures 1,2) using this plasmid and the mRNA (Figures 3,4).

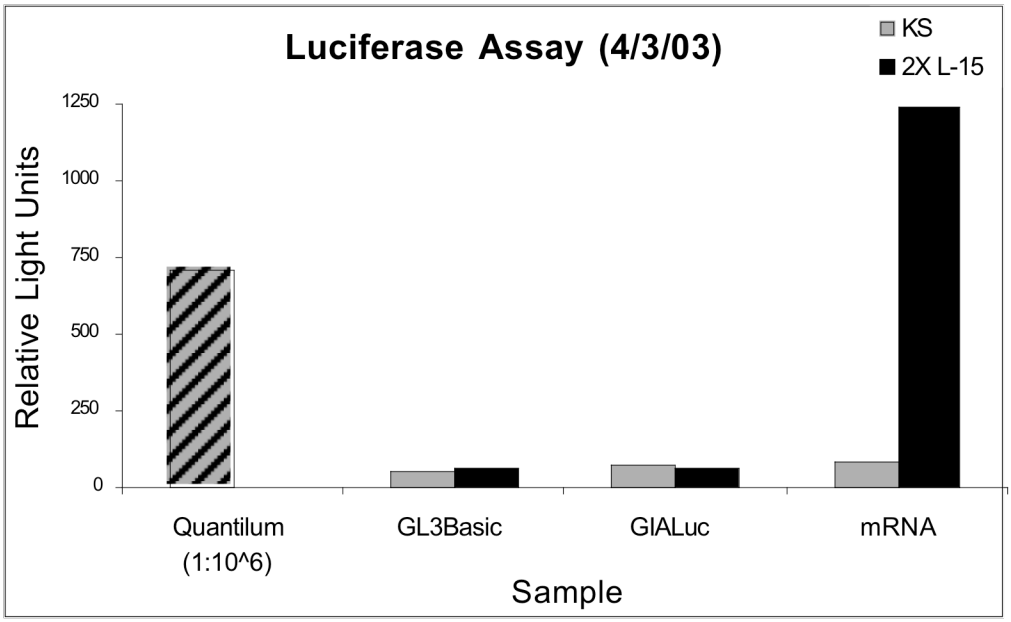


Figure 3. Introduction of transgenes into *C. gigas* primary cells using the GiaLuc (=pGL3-Gia) plasmid, GL3Basic (negative control) and luciferase mRNA in KS and 2xL-15 media. Quantilum reagent was used as a positive control for the luciferase assay. All assays were conducted using the Turner Reporter Microplate Luminometer.

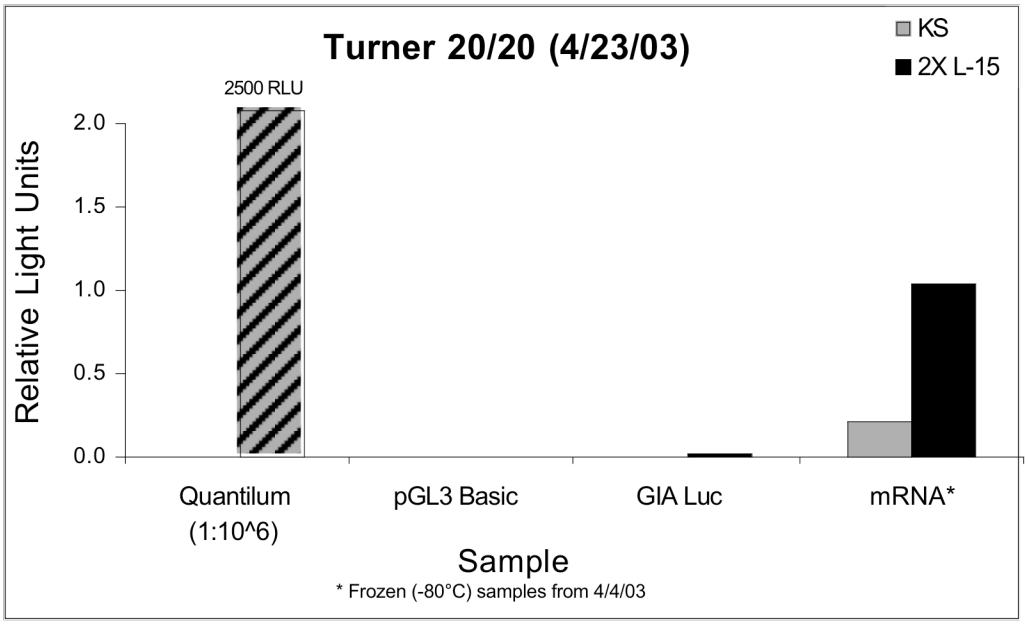


Figure 4. Examination of lipofection efficacy using a Turner 20/20 single tube luminometer and luciferase mRNA, pGL3Basic (negative control) and GiaLuc plasmids. Quantilum reagent was used as a positive control for the luciferase assay.

Assessing the GiaLuc plasmid

Given the difficulty in introducing our reporter transgene into the oyster cells, we examined the ability of introducing GiaLuc and a positive control plasmid, pLuc, into fish cells which are known to accept both plasmids (Burns, pers. comm.). In addition, we examined the plasmid for evidence of insertion of the luciferase gene in the GiaLuc using a restriction enzyme digest and PCR.

To create the GiaLuc plasmid, the oyster actin promoter (Gia) gene (1.5-2 Kb) was inserted into the pGL3-Basic vector (4.8 Kb). Theoretically, if the correct gene was inserted into the vector, the plasmid would be 6.3-6.8 Kb. To determine the size, we digested the plasmid according to the manufacturers instructions with the restriction enzyme *Hind* III (Promega) into one linear piece of DNA. *Hind* III was chosen because there is only one restriction enzyme site on the pGL3-Basic vector. After digestion, the DNA was separated on an agarose gel along side a 1 Kb Plus DNA ladder as a size standard. As expected, an approximately 7 Kb band appeared on the agarose gel (Figure 5).

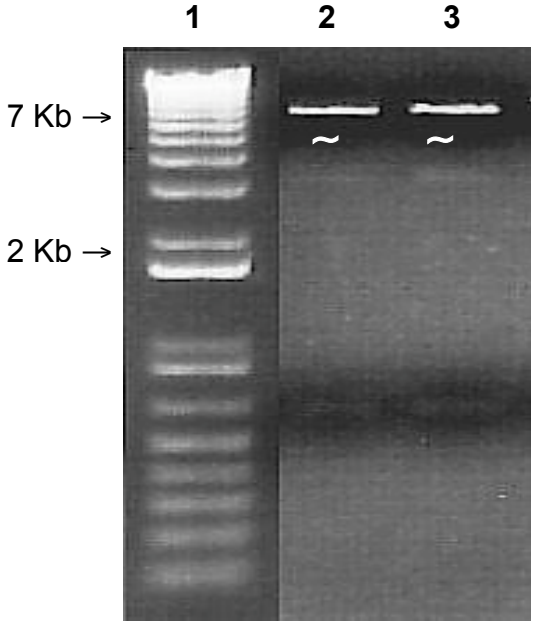


Figure 5. Agarose gel of restriction enzyme digest of pGiaLuc with *Hind* III (Lanes 2 and 3). Lane 1 contains 1Kb Plus Ladder for a size standard. Curved lines represent the where the pGL3-basic plasmid would be on the gel (4.8 Kb), had it been digested with *Hind* III.

We examined the plasmid further with PCR using RV primers 3 and 4. Briefly, each 20 µl reaction contained 10x PCR buffer (10mM Tris, pH 8.3; 50mM KCl), 2.0 mM MgCl2, 10.0 µg BSA, 0.08 mM each dNTP, 0.02 µg each primer, 1 U *Taq* Polymerase (Promega) and 1.0 µl of template DNA. Nucleic acid amplification was performed with an initial denaturation step at 94°C for 2 min, followed by 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final elongation at 72°C for 7 min. Products were separated on 1.0% agarose gels containing 0.1 µg/mL ethidium bromide and visualized using a UV transilluminator. RV primers 3 and 4 bind to the pGL3-Basic sequence reference points 2080-2061 and 4760-4779. PCR of the pGL3-Basic plasmid alone would produce a product of 2.7 Kb. Since the Gia gene is inserted between these reference points, a PCR product between 4.2 and 4.7 Kb was expected. Our gel shows that the amplified PCR products were in this size range (Figure 6).

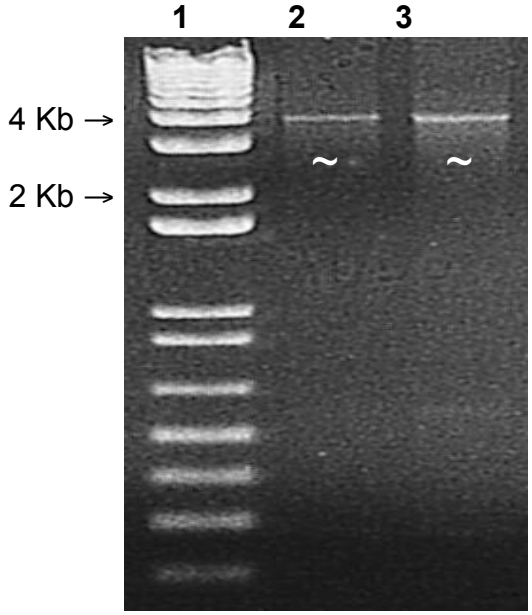


Figure 6. Agarose gel of PCR products generated with the RV primers 3 and 4 and the GiaLuc plasmid (Lanes 2 and 3). Lane 1 contains 1Kb Plus Ladder for a size standard. Curved lines represent the where the PCR products generated with the RV primers 3 and 4 and the pGL3-basic plasmid would be on the gel (2.7 Kb).

BF-2 cell cultures

BF-2 cells, supplied by the Western Fisheries research Center (Seattle WA), are a fibroblastic cell line derived from the caudal fin of bluegill sunfish (*Lepomis macrochirus*) fry. These were used in a transfection assay with GiaLuc and Luc plasmids to assess that the GiaLuc plasmid was intact after unsuccessful transfection assays with the GiaLuc plasmid using our standard protocols. The BF-2 cells were maintained in MEM-10-SB medium (MEM medium supplemented with 10.4% FBS, 1.0% L-glutamine, 1.2% sodium bicarbonate buffer, 16 mg/L gentamycin, 2000 µg/L Fungizone, 100 U/mL penicillin, and 100 µg/mL streptomycin). Once

they were ready to be plated in 24-well cell culture plates and kept in MEM-5-T medium until use. Transfection and reporter gene assays were carried out as described above.

As illustrated in figures 7 and 8 below, introduction of the GiaLuc plasmid was negligible as compared to that of the pLuc plasmid. However, the latter plasmid is designed for use in fish and thus these data are not altogether surprising. However, we did expect more efficient lipofection of the GiaLuc plasmid as evidenced by previous studies. We will next further assess the use of our vectors in concert with one another by comparing luciferase production in oyster and BF-2 cells transfected with the individual plamids and the mRNA alone and cells co-transfected with luciferase mRNA with the GiaLuc plasmid and, separately, with the pLuc plasmid. We expect that the cells into which only the plasmid or only the mRNA alone will produce less luminescence than those co-transfected with the mRNA and plasmid (GiaLuc or pLuc). If successful, this work lays a firm foundation for the next logical step in this research; the Gia promoter and gene of interest (i.e., tachyplesin) would be cloned into the mariner plasmid backbone and co-transfected with a mRNA transposase (ie Tc3A transposase used by Raz et al. 1997) to result in stable integration of the plasmid and gene of interest into the host genome. Once successful, it follows that a next area of investigation lies in assessment of introduction of this plasmid/mRNA combination into embryos or larvae via particle bombardment (gene gun), a method used in gene therapy.

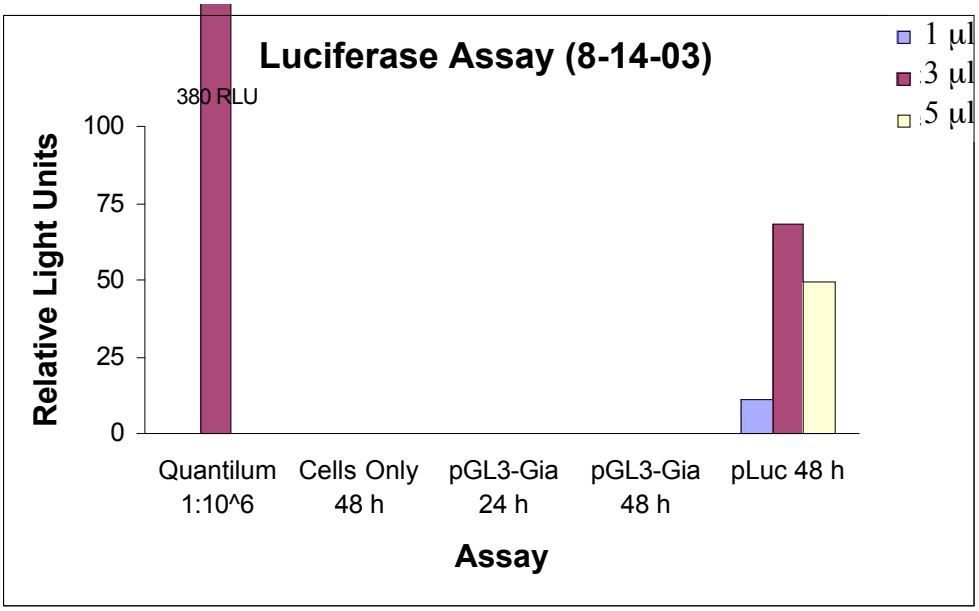


Figure 7. This graph illustrates the successful introduction of the pLuc plasmid into a BF-2 cell line, while the pGL3-Gia plamid was not successfully introduced. Cells without plasmid did not luminesce, while the luciferase control reagent produced a large amount of light. Lipofection was most efficient when 3 µl of Lipofectamine 2000 was employed which is similar to the 5 µl used with the oyster cells.

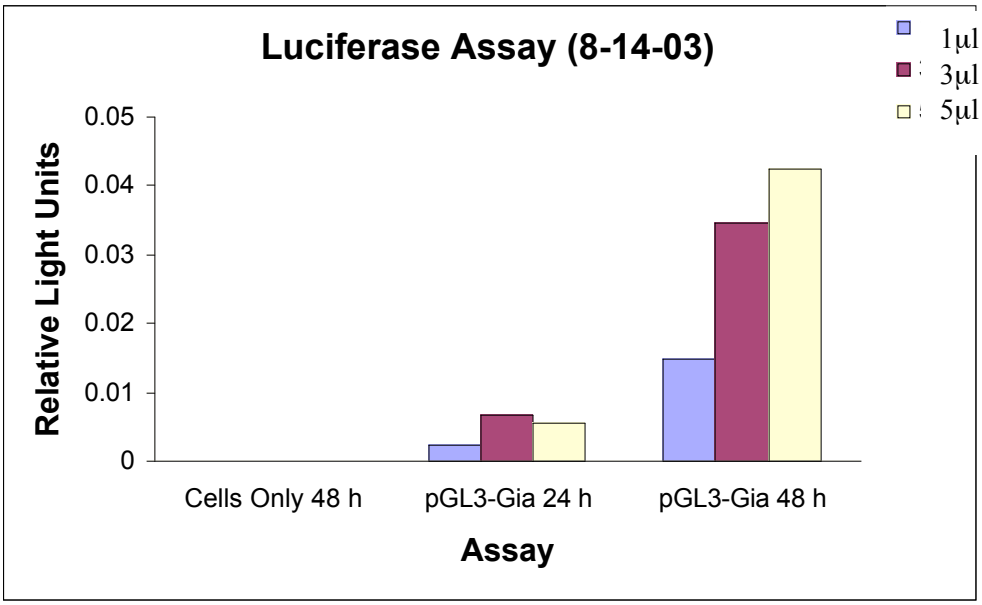


Figure 8. Further examination of BF-2 transfection using the pGL3-Gia (=GiaLuc) plasmid. Introduction of the luciferase gene did appear to occur but the efficiency was very low as compared to introduction of the pLuc plasmid. In this case 5 µl of Lipofectamine 2000 was most effective.

Producing antibody to tachyplesin

A plasmid creating a fusion protein between the prokaryotic maltose binding protein and tachyplesin was constructed. Bacteria were transformed with the plasmid and protein synthesis was induced with IPTG. Protein gel analysis of bacterial lysates suggested that a protein of the correct size was being inducibly expressed in these cultures. The lysate was purified on a maltose-binding column, the MBP was enzymatically cleaved off, and a small amount of mature peptide was obtained and run on a gel to check the size. However, the expense of the enzyme to cleave off the MBP and the tiny yield of this procedure made it clear that a different approach would be necessary. Therefore, mature tachyplesin was synthesized. The purified peptide had the expected antimicrobial activity confirmed by MIC testing against a panel of gram positive and gram negative bacteria. The synthetic peptide was then used to immunize 2 rabbits through the UC Davis core facility.

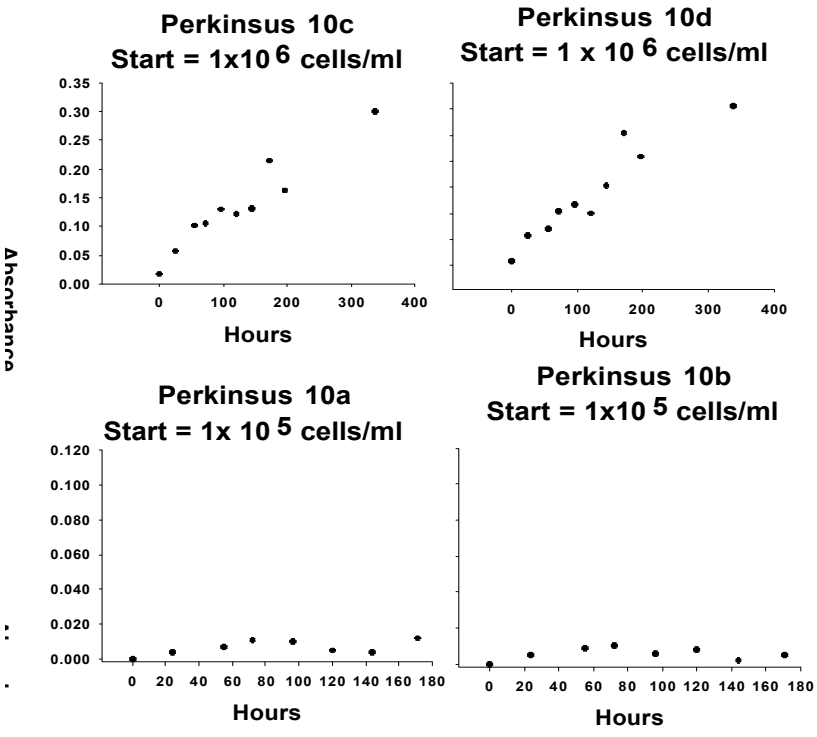
Pre-bleeds and post-bleeds (8 weeks post-immunization protocol) and two subsequent bleeds after boosting were tested by immuno slot blot and Western blotting. Only a very faint band of the correct size was detected using pure, synthetic tachyplesin (1 ug/lane) as the antigen resolved on a Tris-tricine 16.5% gel transferred to an Immobilon P^{sq} membrane. No signal was detected from cell lysates of the pantropic vector producer cell line or from lysates of 293 cells transfected with two plasmids: pLTachyRNL or GIA-Tachy1. Because of the poor quality of the polyclonal antibody, it was not technically feasible to pursue immunodetection of the peptide in the other systems proposed for this grant.

Specific Aim: Test antimicrobial function of expressed peptide against *P. marinus*.

Perkinsus Cultures

Perkinsus marinus cultures were cultured to the log phase of growth, seeded at 1 x 10^6cells/ml into fresh tissue culture plates and incubated at 28EC. Plates were periodically assessed for cell grow using a cell proliferation assay in which cell grow is assess by changes in optimcal density (OD) or by direct cell counts using a hemocytometer (Figure 3). Different *P. marinus* isolates grew differentially as shown in Figure 3. The direct cell count method was less reliable than the cell proliferation assay. The *P. marinus* cells reduced in size as stationary phase approached and, thus, maintaining cells in the log phase of growth was crucial for repeatability of growth assessment. Given the difficulties in introducing transgenes as outlined in the proposal we chose to focus on the development of alternate tools for the introduction of transgenes into oyster cells as described above.

Figure 3: *Perkinsus marinus* cell growth using a cell proliferation assay.



Abstracts:

Boulo V, Cadoret JP, Shike H, Shimizu C, Burns JC. Infection by pantropic retroviral vectors of primary cultured cells from the oyster, *Crassostrea gigas*. Presented at the World Aquaculture Society Meeting, Nice, France, May 2000.

Boulo V, Moore JD, Shimizu C, Friedmann CS, Burns JC. Infection of primary cultured cells from two oyster species by pantropic retroviral vectors. Presented at the Society for In Vitro Biology, San Diego, CA June 2000; In Vitro Cell. Develop. Biol. 36:37A, 2000.

Burns JC, Shimizu C, Boulo V, Shike H. Pantropic retroviral vectors for gene transfer into invertebrate cells. Presented at the Society for In Vitro Biology, San Diego, CA June 2000; In Vitro Cell. Develop. Biol. 36:12-A, 2000.

James D. Moore, Viviane Boulo, Chisato Shimizu, Hiroko Shike, Carolyn S. Friedman, Jane C. Burns, Optimizing culture conditions for creation of an oyster cell line, NSA 2001.