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MARINE BIOTECHNOLOGY

Original Contributions

Transfection of Eastern Oyster (*Crassotrea virginica*) Embryos

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Abstract: There is a need for research in disease resistance and microbial elimination in the eastern oyster *Crassostrea virginica*. Gene transfer may lead to advances in this area, and a means of selecting transfected larvae would be useful. We transfected 3-hour-postfertilization embryos with the bacterial gene aminoglycoside phosphotransferase II (*neo*^{r}), which confers resistance to neomycin and related antibiotics such as G418. The antibiotic G418 was examined as a potential selective agent. A neutral red assay was used to determine survival after 48 hours of exposure to various concentrations of G418 (0–4 mg/ml). We examined the effects of electroporation and chemically mediated transfection of 3-hour-postfertilization embryos on survival to straight-hinge larvae. DNA alone was found to have no effect on survival ($P > .05$). For electroporation we found that increased voltage and pulse duration decreased survival ($P < .05$). Chemically mediated transfection did not significantly affect survival ($P = .5172$). Transgenic larvae were identified after electroporation and chemically mediated transfection. These larvae were reared for 24 hours and exposed to G418 at 0.3 mg/ml for 48 hours. Significant differences in survival between transfected and nontransfected larvae were detected for electroporation ($P = .0147$) and chemically mediated transfection ($P = .037$). Gene transfer was also confirmed with polymerase chain reaction and observation of expression of green fluorescent protein. This study documents the first successful insertion and expression of foreign DNA in eastern oyster larvae.

Key words: *Crassostrea virginica*, gene transfer, antibiotic resistance, green fluorescent protein, transgenic bivalve.

Introduction

In Louisiana over 120,000 hectares of bottom area is privately leased for production of the eastern oyster *Crassostrea virginica* (Keithly et al., 1993). With an annual economic value in the hundreds of millions of dollars in the United States, this estuarine organism comprises an important national fishery (NMFS, 1999). Recently, disease problems from protozoan parasites have plagued this industry (Ford and Tripp, 1996; Paynter, 1996). The transfer of human pathogens (such as *Vibrio vulnificus*) from oysters to human consumers has become a serious concern as well (Jackson et

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al., 1997). The use of hatcheries is common in the culture of bivalves. In these situations bacterial contamination of larval cultures can result in mortality and loss of cultures (Tubiash, 1975; Elston, 1984), especially by outbreaks of larval vibriosis (Brown and Losee, 1978; Ford and Tripp, 1996). Along with research in selective breeding and ploidy manipulation, techniques for gene transfer in the eastern oyster are under investigation as a means of addressing these problems.

Study of gene delivery to commercially important species of finfish and shellfish has been pursued for more than 15 years (reviews by Gong and Hew, 1995; Sin, 1997). There is a large body of research on gene transfer in finfish; however, there are few reports of successful gene transfer in mollusks. Gene transfer has been reported in abalone by electroporation of embryos in red abalone *Haliotis rufescens* (Powers et al., 1995) and electroporation of sperm in Japanese abalone *Haliotis diversicolor* (Tsai et al., 1997). Transgenic dwarf surf clams *Mulina lateralis* were produced using retroviral integration after electroporation of fertilized eggs (Lu et al., 1996). Gene transfer has been reported in the Pacific oyster *Crassostrea gigas,* by particle bombardment of embryos (Cadoret et al., 1997a) and microinjection of fertilized eggs (Cadoret et al., 1997b). Although shellfish are typically highly fecund, the small size of eggs and embryos can be problematic for genetic manipulation. Two methods for gene delivery suitable for working with large numbers of oyster embryos were tested in this study: electroporation and chemically mediated transfection.

Electroporation involves the use of an electric current to create transient pores in the cell membrane, through which DNA can enter the cell (Shigekawa and Dower, 1988). This technique has been used successfully to deliver DNA to embryos and gametes of several aquatic species (reviews by Gong and Hew, 1995; Sin, 1997). Chemically mediated transfection involves the interaction of DNA with various molecules to allow DNA to cross the cell membrane and enter the cell. Cationic lipids, which are often used in this manner to deliver DNA to cells (lipofection) (Felgner et al., 1987), have been used to deliver DNA to embryos of the African catfish *Clarias gariepinus* (Szelei et al., 1994). As an alternative to lipids, synthetic cationic polymers have been developed for DNA delivery. For example, activated polyamidoamine dendrimers (Haensler and Szoka, 1993; Tang et al., 1996) have been used to deliver DNA at high efficiencies in vitro (Kukowska-Latallo et al., 1996). The present study utilized an activated polyamidoamine dendrimer solution available commercially as SuperFect (Qiagen Inc., Valencia, Calif.). There are no reports of the use of activated polyamidoamines to deliver genes to whole organisms.

Not only does the small size of bivalve gametes and embryos make transfection difficult, but selection of transformed larvae becomes difficult as well. There are obvious problems in observing evidence of gene expression in larvae that range in size from 45 to 250 μm. In cell culture transfection of cells with a gene for antibiotic resistance and subsequent treatment with toxic concentrations of antibiotic is an effective method for selecting transfected cells. Development of such a selection method for bivalve larvae would be useful.

One of the genes most commonly used in cell culture for transmission of antibiotic resistance is the aminoglycoside phosphotransferase II (APT) gene (*neo*^{r}), which confers resistance to neomycin and related antibiotics, such as G418. Although bacterial in origin, this gene and its product function effectively in eukaryotic cells, particularly when used for selection with the antibiotic G418 (which inhibits protein synthesis) (Colbere-Garapin et al., 1981; Southern and Berg, 1982). Although commonly used in vitro (Ausebel et al., 1999), this gene has been successfully transferred to organisms.

Evidence of gene expression of *neo*^{r} has been reported in mice (Kaur et al., 1998), fishes (Yoon et al., 1990; Szelei et al., 1994), *Giardia lamblia* (Yu et al., 1996), trypanosomes (ten Asbroek et al., 1990), fruit flies (Steller and Pirrotta, 1985), and tobacco plants (Paszkowski et al., 1992). Successful insertion of *neo*^{r} has been reported for dwarf surf clams (Lu et al., 1996), although gene expression was not documented. At the organismal level, there are few reports of transfection with *neo*^{r}, expression of the gene, and resistance to antibiotic toxicity. In protozoans the unicellular parasites *Giardia lamblia* (Yu et al., 1996) and *Trypanosoma brucei* (ten Asbroek et al., 1990) have been successfully transformed with *neo*^{r} and selected in culture with G418. In multicellular animals the only transfer of antibiotic resistance reported for whole organisms was the selection of transgenic fruit fly larvae with food containing G418 after transfection with *neo*^{r} (Steller and Pirrotta, 1985). Cell lines started from mice transfected with *neo*^{r} have shown resistance to G418 in vitro (Kaur et al., 1998). There are no reports of antibiotic resistance based on *neo*^{r} expression in aquatic species. Attempts at selection with G418 after transfection with *neo*^{r} have been unsuccessful in zebrafish *Danio rerio* (Gibbs et al., 1994) and goldfish *Carrassius auratus* (Yoon et al., 1990).

Another commonly used reporter gene is green fluo-

rescent protein (GFP) (Chalfie et al., 1994). The use of various forms of GFP, originally isolated from the jellyfish *Aequorea victoria,* as reporter molecules is well characterized (Arnone et al., 1997; Higashijima et al., 1997). A modified form of the original GFP isolate, red shifted green fluorescent protein (*rsGFP*), absorbs blue light at a maxima of 490 nm and emits green light at a maxima at 510 nm (Heim et al., 1995). Because this protein does not require any substrates, co-factors, or enzymes for fluorescence, it can be detected in living cells in real time, making it a useful indicator of successful transfection.

The goal of this study was to develop techniques useful for the transfection of eastern oyster embryos and for selection of transfected larvae with antibiotic resistance. Specifically, our objectives were to (1) develop conditions and techniques for gene delivery by electroporation of embryos; (2) develop conditions and techniques for gene delivery by transfection of embryos with polyamidoamine dendrimers (SuperFect); (3) develop conditions and techniques for antibiotic challenge of larvae; and (4) transfect embryos and evaluate larval resistance to the antibiotic G418. In addition, oyster larvae were examined for expression of *rsGFP* as an indicator of successful gene delivery and expression.

Materials and Methods

Oyster Collection

Adult oysters were collected from the Louisiana Gulf Coast from May through September. The oysters were held at the Louisiana State University Aquaculture Research Station in a recirculating system with artificial seawater (ASW) at 15 ppt for at least 5 days before use (Buchanan et al., 1998). This holding period allowed the oysters to recover from transport and acclimate to the conditions in the laboratory.

Embryo Preparation

Embryos were produced using artificial fertilization. Oysters were opened, gametes were extracted, and artificial fertilization was carried out as described previously (Paniagua-Chavez et al., 1998). Artificial fertilization involved the mixture of 2 to 10 million eggs with approximately 500 sperm per egg in a 1-L beaker with ASW. Eggs were examined for cell division as a sign of fertilization at 30 minutes and transferred to a 5-L bucket containing ASW. At 3 hours after fertilization, embryos were concentrated by siphoning water from the top of the bucket and rinsing settled embryos into a beaker, or by screening the contents of the bucket and catching embryos on a 35-μm nylon screen. Percentage of fertilization was calculated as the number of embryos divided by the total number of embryos and unfertilized eggs. Batches with less than 25% fertilization were not used for studies.

DNA Preparation

Plasmid pS65T-C1 was purchased commercially (Clontech Inc., Palo Alto, Calif.) and carries the genes for *neo*^{r} and *rsGFP*. *Neo*^{r} is driven by the simian virus 40 early promoter (SV40) and allows for selection of transfected cells by conferring resistance to the antibiotic G418; *rsGFP* is driven by the immediate-early promoter from human cytomegalovirus (CMV). The protein rsGFP absorbs light at a maxima of 490 nm and emits at a maxima of 510 nm. Thus expression of this protein can be visualized with a fluorescent microscope and a standard fluorescein isothiocyanate (FITC) filter set.

Escherichia coli strain DH5α transformed with pS65T-C1 was grown overnight in Luria-Bertani (LB) broth at 37°C. Plasmid DNA was extracted and purified using a QIAFilter Plasmid Maxiprep kit (Qiagen Inc., Valencia, Calif.), and DNA was dissolved in distilled, deionized water (ddH_2O) and stored at −20°C until use. Concentration and purity of DNA were estimated by spectrophotometry (GeneQuant RNA/DNA Calculator, Pharmacia Biotech, Piscataway, N.J.) and agarose gel electrophoresis (Ausebel et al., 1999).

Experiment 1: Effect of Electroporation on Larval Survival

At 3 hours after fertilization, embryos were concentrated to 10,000 embryos in 0.8 ml of ASW and transferred to 0.4-cm electroporation cuvettes (Bio-Rad Inc., Hercules, Calif.). Embryos were electroporated with a Bio-Rad Gene Pulser I with a Capacitance Extender (Bio-Rad Inc.), which generates an exponential decay-type electrical field. The effects of electrical field strength, electrical field duration, and DNA concentration on embryo survival to straight-hinge larvae were tested. Electrical field strength was reported as a time constant, τ, which represents the amount of time for the generated electrical field to decay approximately 37% ($1/e$). Because τ equals resistance multiplied by capacitance, increasing capacitance increases electrical field duration. To evaluate the effects on survival of field duration, the effects

of capacitance set at 3, 25, and 250 μF, each at 500, 1125, and 2500 V/cm, were tested. To evaluate the effect of field strength, survival after exposure to 500, 625, 700, 875, 1000, and 1125 V was tested at capacitances of 3 and 25 μF. To examine the effect of the presence of DNA, embryo survival was assayed after incubation with and without DNA, and after electroporation at 25 μF and 625 V/cm with and without DNA. All treatments were performed in triplicate.

To assay embryo survival after electroporation, 1000 embryos were transferred to sterile 50-ml centrifuge tubes (Corning Inc., Corning, N.Y.) in 40 ml of ASW. At 24 hours after fertilization, normal embryos would be expected to develop to straight-hinge larvae (Losanoff and Davis, 1963). At 24 hours after electroporation, larvae were fed the marine algae *Isochrysis galbana* (T-iso) at 15,000 cells/ml. At 48 hours after electroporation, larvae were fixed in the tubes with 5% formaldehyde and stored at 4°C. Tubes of fixed larvae were centrifuged for 8 minutes at 200 *g*. The removal of 35 ml of supernatant left the larvae concentrated in 5 ml of ASW. The larvae were resuspended, and two 1-ml aliquots from each sample were transferred to 24-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, N.J.). Straight-hinge larvae have a distinct appearance compared with embryos (Losanoff and Davis, 1963). Survival to straight-hinge larvae was determined by counting the number of larvae in a well with an inverted light microscope at ×100. The percentage of larvae that had been feeding on algae was determined with a fluorescent inverted microscope using a FITC filter set. Larvae that had been feeding fluoresced red from chlorophyll in the gut (Babinchak and Ukeles, 1979). Larval feeding is an indicator of future survival (His and Seaman, 1992).

Experiment 2: Effect of Chemically Mediated Transfection on Larval Survival

At 3 hours after fertilization, 5000 embryos were transferred in 1 ml of ASW to 6-well tissue culture plates. To deliver DNA to the embryos, a solution of plasmid DNA mixed with SuperFect (Qiagen Inc.) was prepared. SuperFect is a commercially available solution of activated polyamidoamine dendrimers (Tang et al., 1996), and the ratio of DNA (mass) to SuperFect (volume) has been shown to influence transfection efficiencies (Kukowska-Latallo, 1996; Qin et al., 1998). For each well of embryos to be transfected, plasmid DNA was diluted in oyster saline solution (0.48 g/L $CaCl_2$, 1.45 g/L $MgSO_4$, 2.18 g/L $MgCl_2 \cdot 6H_2O$, 0.31 KCl g/L, 11.61 g/L NaCl, 0.35 g/L $NaHCO_3$) and mixed with SuperFect for a total volume of 200 μl. The solution was incubated for 10 minutes at room temperature and added to the well of embryos for a final volume of 1.2 ml. The following treatments were investigated for their effect on embryo development to straight-hinge larvae: DNA alone at 5 μg per well, SuperFect alone at 45 μl per well, and DNA-to-SuperFect ratios of 1:3, 1:6, and 1:9 (μg/μl) with 1 or 5 μg of DNA. All treatments were performed in triplicate. Embryo survival to straight-hinge larvae and larval feeding were assayed as in experiment 1.

Experiment 3: Effect of Antibiotic on Larval Survival

This experiment was performed to investigate the effect of the antibiotic G418 (Sigma Chemical Co., St. Louis, Mo.) on developing oyster larvae. At 24 hours after fertilization, 250 larvae were transferred to each well of 6-well tissue culture plates in a total volume of 10 ml of ASW with various concentrations of G418. A preliminary experiment examined concentrations of G418 from 0 to 2 mg/ml in 0.5-mg/ml increments with each treatment in triplicate. A follow-up experiment examined the effect of G418 on a narrower scale from 0 to 0.75 mg/ml. At 48 hours after fertilization, larvae were fed *Isochrysis galbana* at 10,000 cells/ml. At 72 or 96 hours after fertilization, larval survival was estimated using a neutral red assay. Neutral red, a nontoxic vital stain used in plankton viability assays (Crippen and Perrier et al., 1974), has been shown to stain live bivalve larvae (Manzi and Donnely, 1971). To stain live larvae, 200 μl of a 0.5-g/L solution of neutral red was added to each well. Larvae were incubated 6 hours, fixed with 5% formalin, and stored at 4°C for less than 1 week. Before larvae were counted, 100 μl of acid solution (1 N acetic acid and 1 N sodium acetate) was added to each well to produce a red color. Larvae were counted in the wells using an inverted microscope at ×100. Larvae that accumulated the stain in more than 50% of the shell area were counted as alive at the time of fixation. From counts of stained larvae, percentage of surviving larvae was determined. All antibiotic treatments were performed in triplicate.

Experiment 4: Selection of Transfected Larvae with Antibiotic

For antibiotic selection after electroporation, 10,000 embryos at 3 hours after fertilization were electroporated as described above with 10 μg DNA at 25 μF and 650 V/cm.

Control treatments included embryos incubated with 10 μg DNA only and embryos in ASW only. Each treatment was performed in triplicate.

For antibiotic selection after chemically mediated transfection, 5000 embryos at 3 hours after fertilization were transfected with 5 μg of DNA and 15 μl of SuperFect (1:3 ratio) or with 10 μg of DNA and 30 μl of SuperFect (1:3 ratio). Control treatments included embryos incubated with 10 μg of DNA only, embryos incubated with 30 μl of SuperFect only, embryos transfected at a 1:3 ratio with plasmid DNA without *neo*^{r}, and embryos in ASW only. Each treatment was performed in triplicate.

At 24 hours after transfection, approximately 500 larvae from each treatment were transferred per well to 6-well plates with 10 ml of ASW and treated with G418 (<0.5 mg/ml). At 72 or 96 hours after transfection, the neutral red assay was performed as described above and the percentage of survival was calculated.

Because of variation in initial larval survival among experiments, and variation within experiments in estimation of larval concentrations for individual treatments, a survival score was computed as an indicator of larval survival. The number of larvae surviving in wells treated with G418 was divided by the number of larvae surviving in the control (unchallenged) wells for that treatment. For this score, a value above 1.0 indicated that larval survival increased relative to the control well, a value of below 1.0 indicated that larval survival decreased relative to the control well, and a value of 1.0 indicated that larval survival remained the same relative to the control well.

Statistical Analysis

Data were analyzed statistically with the general linear models (GLM) procedure (SAS Inc., Cary, N.C.). A one-way analysis of variance (ANOVA) was used to compare mean values for each treatment within an experiment. For treatments with data reported as a percentage, an arcsin transformation was performed to better approximate a normal distribution (Zar, 1984). A Duncan's multiple range test was used to separate sample means. A significance level of $P < .05$ was used in all statistical analyses.

PCR Detection of Gene Delivery

Polymerase chain reaction (PCR) was performed for detection of gene delivery in the larvae transfected in experiment 4. Larvae were collected on 40-μm nylon mesh and washed twice with ASW. A QIAmp Blood and Tissue Kit (Qiagen) was used to extract DNA from pooled samples of 500 to 1000 larvae, and DNA was eluted into 30 μl of sterile ddH_2O and stored at −20°C. Primers were designed for detection of plasmid pS65T-C1, and PCR was performed as described previously (Buchanan, 1999).

Visualization of Gene Expression

Because pS65T-C1 contained *rsGFP* in addition to *neo*^{r}, gene expression after insertion could be indicated by the observation of green fluorescence in transfected larvae. For larvae transfected in experiment 4, fluorescence microscopy was performed for detection of gene expression. Larvae transfected with and expressing *rsGFP* would be expected to fluoresce green, while control larvae would not be expected to exhibit green fluorescence brighter than background autofluorescence. Larvae were examined with an inverted fluorescent microscope (Zeiss Axiovert 25, Carl Zeiss Inc., Thornwood, N.Y.) for expression of *rsGFP*. Larvae were placed in 24-well tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes, N.J.) for observation and examined for green fluorescence at ×100 and ×200 with standard FITC filter sets. Photographs were taken by chilling larvae to 4°C, causing larvae to remain still.

Results

Experiment 1: Effect of Electroporation on Larval Survival

Electrical pulse duration influenced survival to straight-hinge larvae. Increases in capacitance reduced larval survival (Figure 1). Survival in nonelectroporated controls was 26% ± 2% (mean ± SE). With increasing voltage, a capacitance of 250 μF resulted in a sharp decrease to 33% ± 10% of control larval survival, 25 μF yielded an intermediate decrease to 61% ± 3% of control survival, and 3 μF resulted in a low decrease in larval survival to 74% ± 6% of control larval survival. At 2500 V/cm, there was no survival in any treatments.

The effect of voltage was examined at 125-V/cm increments starting from 500 V/cm with capacitances of 3 and 25 μF. Increasing voltage caused a decrease in larval survival (Figure 2). Embryo survival to straight-hinge larvae in nonelectroporated controls was 48% ± 2%. At 25 μF, survival was 55% ± 12% of control survival at 500 V/cm, 29% ± 4% of control survival at 625 V/cm, and there was no larval

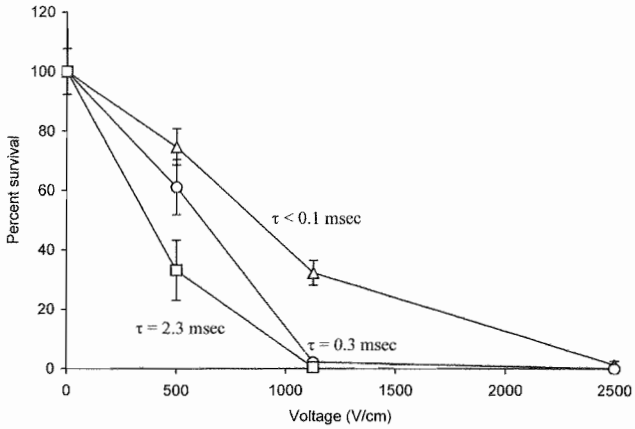

Figure 1. Effect of electroporation on survival of eastern oyster embryos to straight-hinge larvae. Values reported are survival as a percentage of control (mean ± SE). Mean percentage of survival of control samples was 26%. Lines with triangles represent embryos electroporated with a capacitance of 3 μF, circles indicate 25 μF, and squares indicate 250 μF; τ indicates the time constant for that voltage and capacitance.

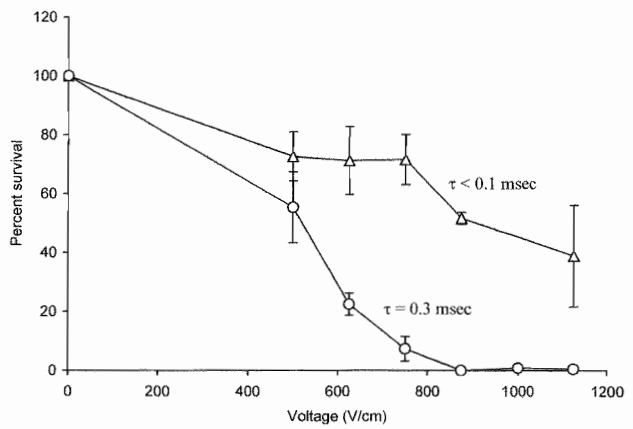

Figure 2. Effect of electroporation on survival of eastern oyster embryos to straight-hinge larvae. Values reported are survival as a percentage of control (mean ± SE). Mean percentage of survival of control samples was 48%. Lines with triangles represent embryos electroporated with a capacitance of 3 μF, circles indicate 25 μF; τ indicates the time constant for that voltage and capacitance.

survival at 875 V/cm. At 3 μF, survival was not affected until 875 V/cm with 52% ± 2% of control survival, and at 1125 V/cm survival was 39% ± 17% of the control.

On the basis of these observations, electroporation of 10,000 embryos in 0.8 ml of ASW at 25 μF and 625 V/cm was selected for further study as these conditions did not induce unacceptable (>50%) mortality. In addition, there were no differences in the percentage of larvae feeding that

Table 1. Results for Feeding Assay for Straight-Hinge Larvae of the Eastern Oyster 48 Hours After Electroporation of Embryos

Capacitance (μF)	Voltage (V/cm)	No. examined	Percent feeding*
0	0	70	89
3	500	79	85
3	625	79	95
3	750	90	87
3	875	90	92
3	1000	91	98
3	1125	56	82
25	500	73	59
25	625	60	79
25	750	22	73
25	875	0	0
25	1000	2	50
25	1125	2	100

*Percent feeding was determined by examination of larvae for red fluorescence from chlorophyll with a fluorescent inverted microscope.

survived electroporation compared with controls (Table 1). This implies that embryos that survived electroporation and developed to straight-hinge larvae were normal.

The effect of DNA on larval survival was tested. It was possible that DNA in solution before or after electroporation could affect larval survival, which would complicate assays for larval survival after electroporation and antibiotic challenge. Therefore, embryos were incubated with and without DNA (pS65T-C1) and electroporated with and without DNA (pS65T-C1). There were no significant differences ($P > .05$) in percentage of survival for control samples with DNA (48% ± 7%) and without DNA (48% ± 1%), or for samples electroporated with DNA (16% ± 1%) and without DNA (14% ± 2%) DNA. Survival was significantly lower in the electroporated treatments ($P < .0001$). In addition, there were no differences in the percentage of larvae feeding after exposure to DNA compared with controls (Table 2). DNA alone was assumed to have no effect on embryo and larval survival for the rest of the study.

Experiment 2: Effect of Chemically Mediated Transfection on Larval Survival

There were no observed effects on embryo survival to straight-hinge larvae ($P = .5172$) after incubation with SuperFect alone, DNA alone, or any of the DNA-SuperFect

Table 2. Results for Feeding Assay for Straight-Hinge Larvae of the Eastern Oyster 48 Hours After Embryo Exposure to DNA and Electroporation

Capacitance (μF)	Voltage (V/cm)	DNA	No. examined	Percent feeding*
0	0	No	70	88
0	0	Yes	88	77
25	250	No	60	78
25	250	Yes	72	72

*Percent feeding was determined by examination of larvae for red fluorescence from chlorophyll with a fluorescent inverted microscope.

(μg:μl) complexes (Figure 3). Increases in the ratio of DNA to SuperFect from 1:3 to 1:9, and increases in DNA concentration from 1 to 5 μg did not decrease larval survival. In addition, there were no differences in the percentage of larvae feeding that survived transfection compared with controls (Table 3). This implies that embryos that survived transfection to develop to straight-hinge larvae were normal. For transfection of embryos in further experiments, conditions chosen for chemically mediated transfection were incubation of 5000 embryos in 1 ml of ASW with 5 μg of DNA and 15 μl of SuperFect, or with 10 μg of DNA and 30 μl of SuperFect (1:3 ratio).

Experiment 3: Effect of Antibiotic on Larval Survival

In a preliminary study, a decrease in pH was observed from approximately 8.0 at 0 mg/ml of G418 to approximately 7.0 at 1 mg/ml of G418, making the use of antibiotic concentrations above 1.0 mg/ml problematic (data not shown). When the effect of the antibiotic on survival from 0 to 0.75 mg/ml was examined, at low concentrations of the antibiotic (0.1 mg/ml) survival was increased to 215% of control survival. However, survival decreased (53% of control survival) as antibiotic concentration increased to 0.25 mg/ml and higher (Figure 4). This trend of increased larval survival at low concentrations of G418 and decreased survival at higher concentrations was repeatedly observed.

From these results, the concentration of antibiotic chosen for challenge of transfected larvae was 0.3 mg/ml. It should be noted that G418 was not only toxic to larvae, but also inhibited growth. Although not quantitated, larvae in all wells receiving the antibiotic were noticeably smaller than control larvae, even when larval survival was enhanced by low concentrations of the antibiotic.

Experiment 4: Selection of Transfected Larvae with Antibiotic G418

Results of antibiotic challenge clearly showed increased survival for larvae electroporated with *neo*^{r} (Figure 5). For low levels of G418, no differences were detected with larvae incubated at 0.1 mg/ml of G418 ($P = .6042$). However, with an antibiotic concentration of 0.25 mg/ml, there were significant differences in survival ($P = .0147$). Survival was significantly higher in the treatment electroporated with DNA (survival score = 1.92).

Antibiotic challenge of larvae transfected with SuperFect and *neo*^{r} also showed increased survival (Figure 6). No differences in survival among treatments were detected with larvae cultured in 0.1 mg/ml of G418 ($P = .4059$). However, with increased antibiotic concentration (0.3 mg/ml), there were significant differences in survival ($P = .037$). The highest levels of survival were observed in embryos transfected with 5 μg of DNA with *neo*^{r} and 15 μl of SuperFect (1:3 ratio) (survival score = 1.26). Intermediate levels of survival were observed in embryos transfected with 10 μg of DNA and 30 μl of SuperFect (1:3 ratio) (survival score = 0.89). Survival was lower and not significantly different in all other treatments.

PCR Detection of Gene Insertion

The presence of *rsGFP* was detected in all samples transfected with plasmid pS65T-C1 (which contained both *rsGFP* and *neo*^{r}). The presence of *rsGFP* was also detected in samples incubated with plasmid DNA. Although band intensity was not quantitated, bands from these reactions were faint and noticeably less intense than bands from samples transfected with *rsGFP* and SuperFect. All other samples were negative for the presence of *rsGFP*.

Visualization of Gene Expression

The expression of *rsGFP* was observed in straight-hinge larvae, although these larvae were rare (<1 in 100). Larvae expressing *rsGFP* were observed only in samples transfected with plasmid pS65T-C1 (containing both *rsGFP* and *neo*^{r}) by electroporation (Figure 7) and chemically mediated transfection. Larvae fluorescing green were not observed in any other samples, including control samples and samples that were only incubated with plasmid DNA. Distribution

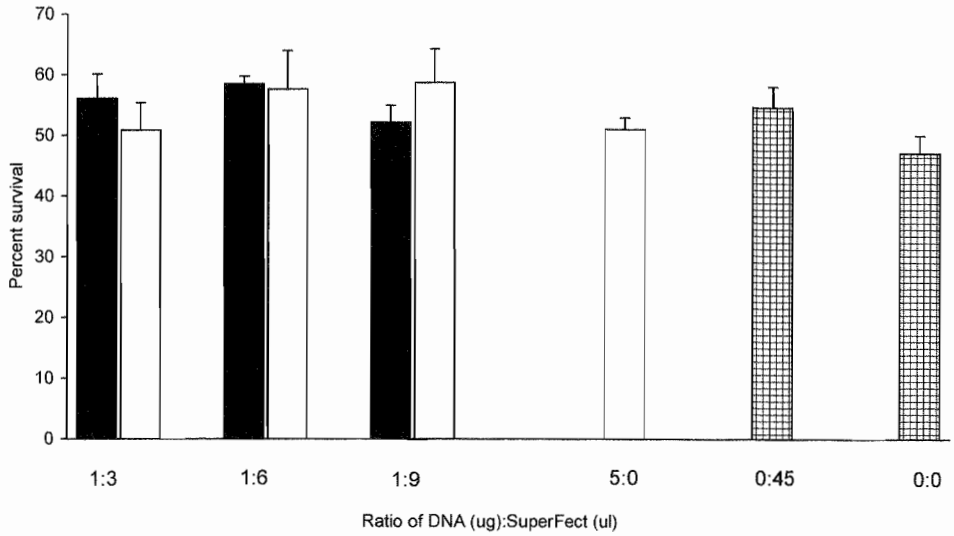

Figure 3. Effect of chemically mediated transfection on percentage of survival (mean ± SE) of eastern oyster embryos to straight-hinge larvae. No significant differences were detected among treatments (P = .5172). Ratios indicate the proportion of DNA (μg) to SuperFect (μl). Embryos received 1 μg of DNA (solid bars), 5 μg of DNA (open bars), or no DNA (hatched bars).

Table 3. Results for Feeding Assay for Eastern Oyster Straight-Hinge Larvae 48 Hours After Transfection of Embryos with DNA Complexed with SuperFect

DNA (μg)	SuperFect (μl)	No. examined	Percent feeding*
1	3	76	90
1	6	52	90
1	9	98	95
5	15	99	94
5	30	78	94
5	45	95	83
5	0	77	87
0	45	71	94
0	0	62	97

*Percent feeding was determined by examination of larvae for red fluorescence from chlorophyll with a fluorescent inverted microscope.

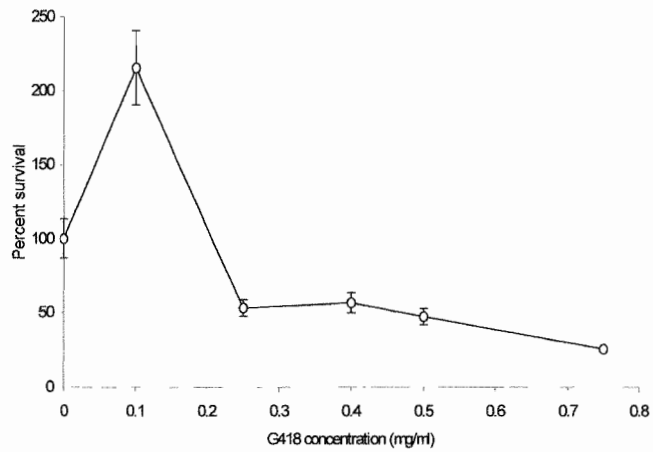

Figure 4. Toxicity of the antibiotic G418 to straight-hinge larvae of the eastern oyster 24 hours after fertilization. The values are reported as survival as a percentage of control (mean ± SE). Mean percentage of survival of control samples was 15%.

of green fluorescence appeared uniform throughout the tissues of larvae in which *rsGFP* expression was detected. Expression of *rsGFP* was not associated with any obvious larval mortality.

Discussion

In this study techniques were developed for the transfection of eastern oyster embryos and selection of larvae. To our knowledge this is the first report of gene delivery to eastern oyster embryos and of electroporation or the use of activated polyamidoamine dendrimers to transfect bivalve embryos, although Lu et al. (1996) did use a combination of electroporation and retroviral integration to transfect fertilized eggs of dwarf surf clams.

For electroporation it is accepted that the degree of cell permeation depends on the field strength and duration of the electric pulse. Higher field strengths or longer pulse durations result in increased permeation (Anderson et al., 1991). However, increased cell permeability is associated with increased cell death (Shigekawa and Dower, 1988). Although there is a positive correlation between the amount of cell death and maximum transfection efficiency for electroporation, the optimum field strength and time constant

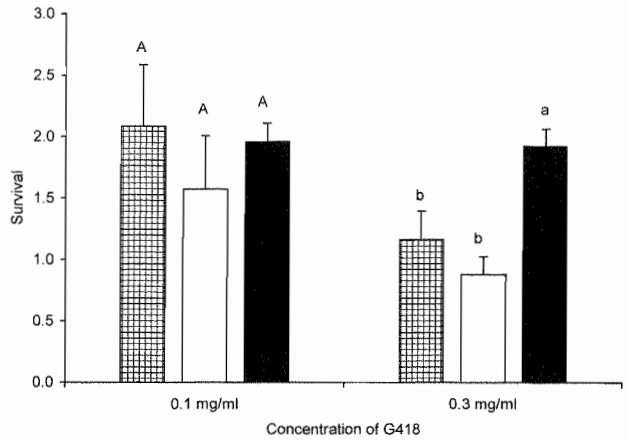

Figure 5. Survival (mean ± SE) of electroporated and potentially transgenic (with *neo*^{r}) eastern oyster larvae after exposure to the antibiotic G418. Bars sharing letters were not significantly different ($P > .5$). Columns with uppercase letters were analyzed separately from columns with lowercase letters. Hatched bars represent samples incubated with 10 μg DNA, open bars were controls, and solid bars were electroporated with 10 μg DNA at 625 V/cm and a capacitance of 25 μF. The time constant, τ, was 0.3 ms.

to produce transfection efficiency must be experimentally determined for each cell type (Shigekawa and Dower, 1988; Anderson et al. 1991).

In human lymphoid cells, maximum transfection efficiency was observed with 18% survival (of controls) after electroporation (Anderson et al., 1991). In this study, electroporation conditions were selected that affected overall embryo viability (approx. 20% survival), but still allowed embryo survival to straight-hinge larvae. These conditions, 625 V/cm, 25 μF, and a time constant of 0.3 ms, were comparable to electroporation with an exponential decay wave of finfish eggs and embryos (Inoue et al., 1990; Buono and Linser, 1992), although the pulse lengths reported here were shorter owing to the lower resistance of the electroporation media (ASW). There are few reports of the use of electroporation to transfect marine bivalves or mollusks (Powers et al., 1992; Lu et al., 1996). These reports used a square wave electrical pulse for cell permeation, and thus the conditions were not directly comparable to those in the present study. When a square wave electrical pulse was used in the red abalone *Haliotis rufescens,* after electroporation at 10,000 V of fertilized eggs, survival to larval stages was reported to be 70% to 84% that of nonelectroporated controls, with 90% of larvae showing transgene integration and 72% showing transgene expression (Powers et al., 1995). These levels of survival are higher than those observed in eastern oysters in this study. In dwarf surf clams, again with a square wave electrical pulse, after electroporation at 500 to 700 V and retroviral integration into fertilized eggs, survival to adulthood was reduced from 15% to 5%, with one third of the surviving adults found to be positive for transgene incorporation by dot blot hybridization (Lu et al., 1996). More efficient electroporation conditions for gene transfer to oyster embryos may exist, but the conditions tested here resulted in transgene delivery and expression.

There are no reports of the use of polyamidoamine dendrimers for in vivo transfection of organisms. Information from in vitro transfection of cell cultures suggests that polyamidoamines can be toxic (Kukowska-Latollo et al., 1996; Tang et al., 1997); however, they did not exhibit toxicity in oyster embryos at the levels we tested. Higher concentrations of DNA and SuperFect could prove more toxic to embryos, yet be more efficient at transfection. However, the conditions tested here resulted in gene delivery and expression.

Suggested concentrations of G418 to use in cell culture for selection of transfected cells range from 0.1 to 0.8 mg/ml (Ausebel et al., 1999). Values in this range were useful for challenge of eastern oyster larvae in the present study. At low concentrations of the antibiotic (0.1 mg/ml), larval survival was enhanced. This is not surprising as low concentrations of antibiotics have proved to be beneficial to bivalve larvae culture (Hidu and Tubiash, 1964; Fitt et al., 1992). Higher concentrations of G418 did result in significantly decreased survival of larvae, though these concentrations did not result in complete mortality of control oyster larvae. Groups of larvae that were transfected with *neo*^{r} demonstrated significantly increased survival in G418 compared with challenged controls. However, these larvae were observed to be smaller than unchallenged control larvae.

Several explanations are possible. The antibiotic G418 is known to decrease growth as well as survival. In cell culture a positive correlation among the level of *neo*^{r} expression and the percentage of survival and growth rates of cells in G418 has been reported (Emerman and Temin, 1984; Paludan et al., 1989; Niwa et al., 1991). Low levels of *neo*^{r} expression in oyster larvae may have allowed survival in G418, but with reduction in growth. Little is known of heterologous promoter function in eastern oysters, and if the SV40 promoter used in this study functions inefficiently in oyster cells, survival and growth would be impacted. In *Drosophila* larvae transfected with *neo*^{r} under control of the *herpes thymidine kinase* (*tk*) promoter were able to survive G418 selection, but at a slower growth rate and with in-

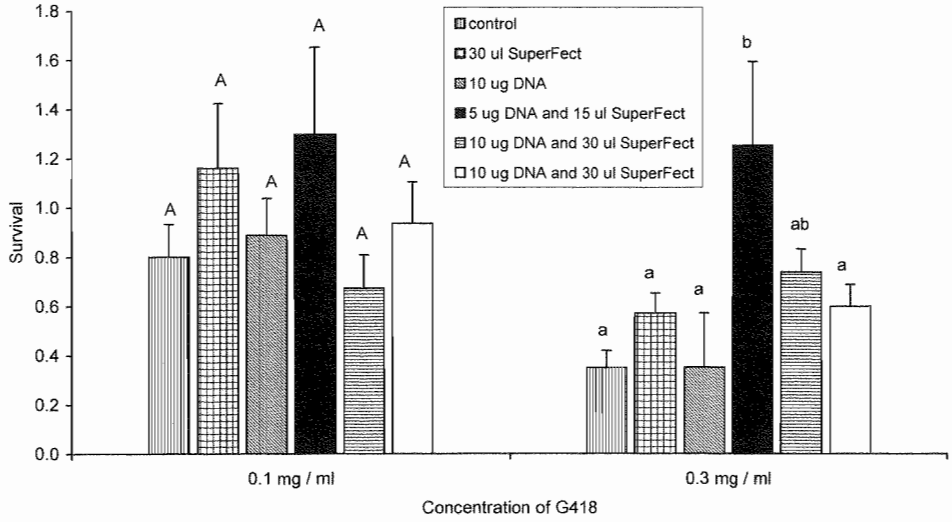

Figure 6. Survival (mean ± SE) of potentially transgenic (with *neo*^{r}) eastern oyster larvae after chemically mediated transfection and exposure to the antibiotic G418. Columns sharing letters were not significantly different ($P > 0.5$). Columns with uppercase letters were analyzed separately from bars with lowercase letters. Treatment with open bar received DNA without *neo*^{r}.

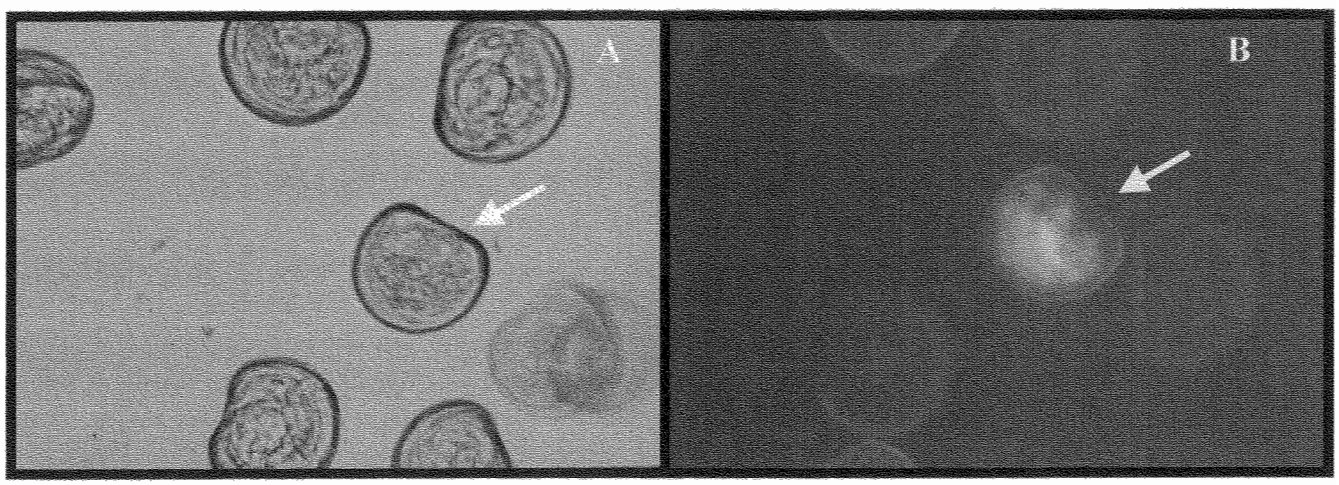

Figure 7. Expression of the gene for red-shifted green fluorescent protein (*rsGFP*) in live straight-hinge larvae of the eastern oyster produced by electroporation of embryos 3 hours after fertilization with pS65T-C1: brightfield micrograph (note movement of rightmost larva) **(A)**; fluorescent micrograph **(B).** Larva indicated by arrow was expressing *rsGFP.* Micrographs were taken with an inverted microscope at ×400.

creased mortality compared with unchallenged control larvae. The *tk* promoter was thought to be weakly expressed, and the transfection of *Drosophila* larvae with the *Drosophila hsp70* heat shock promoter and *neo*^{r} resulted in larval survival and growth rate on G418 equal to those in controls (Steller and Pirrotta, 1985).

In other bivalves mammalian viral promoters have been shown to function, such as the Moloney murine leukemia virus (MMLV) and the Rous sarcoma virus (RSV) promoters in dwarf surf clams (Lu et al., 1996), and the CMV and SV40 promoters in the Pacific oyster (Boulo et al., 1996; Cadoret et al., 1997a, 1997b). The SV40 promoter drove chloramphenicol acetyltransferase (CAT) production in Japanese abalone (Tsai et al., 1997). However, the *Drosophila hsp70* heat shock promoter was shown to produce higher levels of gene expression in cells of Pacific oysters than did viral promoters, including SV40 (Boulo et al., 1996). Of course, promoter function cannot be known until it is tested, but different promoters should be examined for higher levels of gene expression in eastern oysters.

The level of gene expression depends not only on promoter function, but also on gene copy number. Increased delivery of copies could increase gene expression and survival under selection. However, high levels of *neo*^{r} gene

expression can cause problems. Although transfection of larvae with *neo*^{r} did not affect embryo survival at 24 hours, *neo*^{r} can influence larval development and growth. In cell culture *neo*^{r} expression has been shown to cause changes in cell metabolism, to modify transcription factors, and to effect endogenous gene expression, probably through phosphotransferase activity (Valera et al., 1994). Mice transfected with *neo*^{r} and producing APT were found to be 25% smaller than littermates (Kaur et al., 1998). Therefore, high levels of production of APT could inhibit oyster larval growth and cause increased mortality. In the present study, there were no observed effects on growth rate of transfected larvae grown without antibiotic G418, or on survival of these larvae after 4 days, eliminating APT activity as a causative agent in reduced growth rates and increased mortalities. However, the negative consequences of increased APT production should be considered as improvements in transfection and expression are pursued.

A final explanation for reduced larval growth rate and survival in G418 is mosaicism. Because these embryos were transfected after fertilization and first cell division, it is possible that not all cells received the transgene. Also, the promoter driving gene expression must be expressed in all larval tissues. These factors would allow partial resistance to antibiotic selection, with an associated decrease in growth and increase in mortality. Problems with mosaicism were suggested in poor survival in G418 for *Drosophila* larvae (Steller and Pirotta, 1985) and in failure of G418 selection in transfected goldfish (Yoon et al., 1990).

Further evidence of gene transfer in oyster embryos was documented with observation of *rsGFP* expression after transfection into oyster embryos. Successful transfer and gene expression of *rsGFP* implies successful transfer of *neo*^{r} as well. Although larvae expressing *rsGFP* were observed, the proportion of positive larvae was less than 1%. It is possible that more larvae were expressing this gene below threshold detection levels. The CMV promoter is known to function in the Pacific oyster at lower levels compared with other promoters (Boulo et al., 1996; Cadoret et al., 1997a). If this promoter was not efficient in the eastern oyster, low levels of gene expression would result. In addition, expression of *neo*^{r} can reduce levels of gene expression, especially when both genes are included on the same transfection plasmid (Artlet et al., 1991). Another possibility is simply that the transfection efficiency was low. This seems unlikely given the resistance of a majority of the transfected larvae to G418. From PCR, the true efficiency of transfection is not known at this time. Because of the small size of oyster larvae, PCR for transgene detection was performed on pooled samples of 500 to 1000 larvae. However, the data from delivery of *rsGFP* to embryos is clear evidence of successful gene transfer and gene expression in oyster larvae.

It was noted that the incubation of embryos with DNA resulted in larvae positive with PCR for plasmid DNA (pS65T-C1). This is not surprising as cells can readily take up DNA from solution (Bennet et al., 1993), although usually at reduced efficiencies compared with techniques for gene delivery. Alternatively, these bands may have resulted from small amounts of contaminating DNA present after washing larvae before DNA extraction. These PCR bands were faint and obviously less intense than bands from larvae transfected with DNA and SuperFect, implying that DNA delivery was higher in larvae transfected with DNA and SuperFect. No larvae expressing *rsGFP* were observed from embryos incubated with DNA only. If these larvae received DNA that was detected by PCR, it was probably at a low copy number, precluding high levels of *rsGFP* expression and observation of green fluorescence as well as efficient expression of *neo*^{r}.

The goal of this work was to demonstrate transfection and selection of eastern oyster embryos. Although transfection was successful and increased survival for transfected larvae exposed to G418 was documented, further modification of techniques is necessary for selection. To increase the utility of G418 as a selective agent, the use of buffers to eliminate pH effects on larval survival will have to be investigated at higher doses of the antibiotic, as will the effects of long-term larval exposure to G418. Further work is needed for identification of more useful promoters for use in eastern oysters. Molluscan promoters have been identified recently (Yoshino et al., 1998; Cadoret et al., 1999), and *Drosophila* promoters have been shown to function well in molluscan cells (Powers et al., 1995; Boulo et al., 1996). Although improvements in technique will be necessary for selection of transgenic larvae, this study documents successful gene delivery to *C. virginica* embryos by electroporation and chemically mediated transfection, the expression of *rsGFP* in larvae, and the expression of *neo*^{r} with subsequent increased larval survival in the presence of G418.

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