Electrotransfer of the epinecidin-1 gene into skeletal muscle enhances the antibacterial and immunomodulatory functions of a marine fish, grouper (Epinephelus coioides)

Lin-Han Lee\textsuperscript{a}, Cho-Fat Hui\textsuperscript{b}, Chi-Mu Chuang\textsuperscript{c,d}, Jyh-Yih Chen\textsuperscript{a,}\*  

\textsuperscript{a}Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, 23-10 Dahuen Road, Jiaoshi, Ilan 262, Taiwan  
\textsuperscript{b}Institute of Cellular and Organismic Biology, Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei 115, Taiwan  
\textsuperscript{c}Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Taipei Veterans General Hospital, Taipei, Taiwan  
\textsuperscript{d}Faculty of Medicine, School of Medicine, National Yang-Ming University, Taipei, Taiwan  

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\textbf{A B S T R A C T}  
Electrotransfer of plasmid DNA into skeletal muscle is a common non-viral delivery system for the study of gene function and for gene therapy. However, the effects of epinecidin-1 (epi) on bacterial growth and immune system modulation following its electrotransfer into the muscle of grouper (Epinephelus coioides), a marine fish species, have not been addressed. In this study, pCMV-gfp-epi plasmid was electroporated into grouper muscle, and its effect on bacterial numbers at 24 and 48 h after infection, and augmented the expression of immune-related genes in muscle and liver, inducing a moderate innate immune response associated with pro-inflammatory infiltration. Furthermore, electroporation of pCMV-gfp-epi plasmid without V. vulnificus infection induced moderate expression of certain immune-related genes, particularly innate immune genes. These data suggest that electroporation-mediated gene transfer of epi into the muscle of grouper may hold potential as an antimicrobial therapy for pathogen infection in marine fish.

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1. Introduction

The delivery of plasmid DNA encoding functional protein via injection into skeletal muscle has the potential to treat an extensive range of diseases [1–3]. The factors affecting gene transfer efficiency and recombinant protein expression following gene delivery include: (i) the system of delivery, (ii) the immune response to the plasmid or transgenic protein, and (iii) non-specific expression of the transgene in non-muscle cells. The electroporation method of delivering plasmid DNA is based on the application of an electric field, to both enhance cell permeability and provide an electrostatic force to introduce the plasmid DNA [4]. Electroporation enhances uptake of plasmid DNA by skeletal muscle by more than 2000-fold, as compared to injection of plasmid DNA alone; furthermore, expression of exogenous DNA can persist for up to one year [5]. Enormous improvements in the technology and transduction efficiency in mammalian systems have been made over the past decade, and it has now been successfully used in pre-clinical and clinical trials for the transfer of therapeutic plasmids and DNA vaccines [6–8].

Intra-muscular injection of plasmid DNA has been studied in several fish species, including carp, tilapia, goldfish, zebrafish, and flounder [9]. However, to date there are few reports on the electroporation of fish muscle [10–13]. Intra-muscular injection combined with electrotreatement of plasmid DNA has only thus far been reported for zebrafish and European seabass (Dicentrarchus labrax) [10,11]. The efficiency of gene transfer following intra-muscular injection of plasmid DNA is relatively poor in marine as compared to freshwater fish, on account of differences in muscle plasma osmolality and ion concentrations. It has been shown that transfection efficiency in a mammalian system can be enhanced by using a high voltage electric pulse [14], but differences in plasma osmolality may make this unsuitable for marine fish.

Antimicrobial peptides (AMPs) are key components of innate immunity in all multicellular organisms, and their bactericidal properties have been extensively studied [15,16]. Their mechanism of action involves permeation of bacterial membranes, which is presumed to reduce the possibility of the emergence of AMP
resistance [17]. The bactericidal activity of a substantial number of marine organism AMPs against different pathogens have been examined in vitro [18]. In addition, it has been shown that injection of zebrafish muscle with AMP-encoding plasmids significantly enhanced bactericidal ability when combined with electroporation [10]. More recently, epinecidin-1 (epi), a synthetic peptide from grouper (Epinephelus coioides), was reported to be effective for the treatment of Vibrio vulnificus-induced bacteremia in fish [19]. Epi also has bactericidal activity against Gram-negative and -positive bacteria, viruses, Candida albicans, and Trichomonas vaginalis [20–22]. These results strongly suggest that epi may be a suitable alternative to antibiotics in aquaculture. In this study, it was investigated whether administration of epi via electroporation of muscle counters V. vulnificus 204 infection in grouper.

2. Materials and methods

2.1. Fish, plasmid, and bacterial strains

Female grouper (E. coioides) with a body length of about 5 cm were bought from a local fish farm, and cultured in a 4000 l indoor aquarium at the Marine Research Station, Ilan, Taiwan. The aquarium was supplied with constant flow of sea water (salinity 30–33‰) and maintained at room temperature (25–28 °C). The fish were fed on a commercial diet during the acclimatization. After the acclimatization period, the weight and body length of each grouper was measured, and grouper were randomly divided into experimental tanks (4 fl; 10 fish per tank). The construction of the CMV-gfp-epi plasmid has been previously described [10]. The V. vulnificus (strain 204) was a gift from Dr. Chun-Yao Chen (Tsui Chi University, Hualien, Taiwan). Fish pathogens used in this study were cultured at 27 °C. All fish care protocol and handling procedures were in accordance with Academia Sinica guidelines. Experiments using fish were performed according to “The Ethical Guideline for Using Vertebrates as Experimental Animals in Taiwan”, and were approved by the “Ethical Committee for Using Vertebrates as Experimental Animals” of Academia Sinica.

2.2. Intramuscular injection, “in vivo” electroporation, RNA extraction, and quantitative reverse transcription PCR (qRT-PCR)

The CMV-gfp-epi plasmid was injected into the mid-region of the muscle of five grouper (body length 5.66 ± 0.43 cm, body weight 3.27 ± 0.23 g) (Supplementary Fig. 1). Tweenzzer type electrodes (Super Electroporator NEPA21, NEPA GENE CO., LTD, Chiba, Japan) were used to electroporate DNA into grouper muscle. Grouper were temporarily removed from the water and given an intramuscular injection of a solution of plasmid DNA (approximately 20 µl) at the muscle on one flank of the fish. Fish were returned to the aquarium immediately after injection.

The optimal conditions for each parameter of electroporation were determined. The optimal voltage was identified using a pulse number of 2, 10 µg of injected plasmid, and a duration of 96 h. Optimal pulse number was determined using a voltage of 50 V, 10 µg of injected plasmid, and a duration of 96 h. Optimal duration was determined using a voltage of 50 V, a pulse number of 2, and 10 µg of injected plasmid. Optimal DNA concentration was determined using a duration of 48 h, a pulse number of 2, and a voltage of 50 V. After the elapsed duration, the levels of epi mRNA were measured by quantitative reverse transcription (RT)-PCR (qRT-PCR). RNA extraction and reverse transcription were performed as previously described [10]. Levels of mRNA were quantified using comparative RT-PCR [23]. The final volume of the RT-PCR mixture was 10 µl, and RT-PCR was performed using the following conditions: 95 °C for 20 s; 40 cycles of 95 °C for 3 s and 60 °C for 30 s; 95 °C for 15 s; 60 °C for 60 s; and finally 95 °C for 15 s. Mixtures without template or primers served as negative controls. Expression of egfp under each experimental condition was normalized to internal levels of β-actin, and data are presented as multiples of the increase relative to an arbitrary value of 1. Ten fish were used for each experiment, and each data point represents the mean value from at least five fish.

2.3. DNA extraction and PCR

Genomic DNA was extracted from the muscles of experimental and control fish at 48 h after electroporation, as described previously [10]. Genomic DNA was also extracted from muscle, heart, liver, gill, spleen, kidney, and intestine. Briefly, tissues were frozen in liquid nitrogen and homogenized using a mortar and pestle. The resulting homogenates were dissolved in extraction buffer (10 mM Tris–HCl, 100 mM EDTA, 20 µg/ml pancreatic RNAase, 0.5% SDS, pH 8.0) for 16 h at 50 °C with 0.1 mg/ml proteinase K. Undissolved tissue was removed by centrifugation, and DNA samples were extracted with phenol (equilibrated with Tris–HCl, pH 8.0) and precipitated with ethanol. The genomic DNA from each tissue was used as template to detect the plasmid by PCR, under the following conditions: 95 °C for 5 min; 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and 72 °C for 5 min. Samples were then maintained at 4 °C prior to electrophoresis. The primer sequences are listed in Table 1. The PCR products were analyzed by electrophoresis on an agarose gel and subsequent EtBr staining.

2.4. Evaluation of the bactericidal and immunomodulatory effects of epinecidin-1 in marine fish

Bacterial counts and immune-related gene expression in grouper under various experimental conditions were analyzed. Plasmid DNA (CMV-gfp-epi or CMV-gfp) was electroporated into grouper muscle, and 20 µl of V. vulnificus (strain 204; 2.75 × 10^6 cfu/ml) was injected into muscle 48 h later. Control groups were injected with one of the above plasmids, but were subsequently uninfected with V. vulnificus or injected with PBS instead. A further

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Table 1

Sequences of primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grouper – β-actin-5’</td>
<td>5’ - CCATCAGCGCGTGTTGTCG</td>
</tr>
<tr>
<td>Grouper – β-actin-3’</td>
<td>5’ - AGGAGGAGCGCTTGAAAGA</td>
</tr>
<tr>
<td>Grouper – MyD88-5’</td>
<td>5’ - TATGTCCTACTCTGCTGCCC</td>
</tr>
<tr>
<td>Grouper – MyD88-3’</td>
<td>5’ - ACCATCGGTCTACCTTCCTTC</td>
</tr>
<tr>
<td>Grouper – IL-15-5’</td>
<td>5’ - CGACTGTTGCTGGTTTCTCTT</td>
</tr>
<tr>
<td>Grouper – IL-15-3’</td>
<td>5’ - CTTCGCTGTCGATCAGGAGTT</td>
</tr>
<tr>
<td>Grouper – TNF-5’</td>
<td>5’ - TCTCCATGGCCCTCTTTGTTTA</td>
</tr>
<tr>
<td>Grouper – TNF-3’</td>
<td>5’ - CTTCCATGGCCCTCTTTGTTTA</td>
</tr>
<tr>
<td>Grouper – TNF2-5’</td>
<td>5’ - GCTGGCGGCTGAAAGACAT</td>
</tr>
<tr>
<td>Grouper – TNF2-3’</td>
<td>5’ - CAGAGGTCGGTGATTGAG</td>
</tr>
<tr>
<td>Grouper – NF-κB-5’</td>
<td>5’ - TCCCTACCATGTTGAAAGAC</td>
</tr>
<tr>
<td>Grouper – NF-κB-3’</td>
<td>5’ - CCTCAGACCTTGACATG</td>
</tr>
<tr>
<td>Grouper – MX-5’</td>
<td>5’ - AACAGACCTGACACAC</td>
</tr>
<tr>
<td>Grouper – MX-3’</td>
<td>5’ - ATGAGGTTGCTCGCAC</td>
</tr>
<tr>
<td>Grouper – IL-8-5’</td>
<td>5’ - TGTACATCCTCATGCCC</td>
</tr>
<tr>
<td>Grouper – IL-8-3’</td>
<td>5’ - AACTCTTGGCTGCTC</td>
</tr>
<tr>
<td>Grouper – RF1-5’</td>
<td>5’ - TCTCAGTACATAGACACAC</td>
</tr>
<tr>
<td>Grouper – RF1-3’</td>
<td>5’ - TGGCTTCTGACCTGCTG</td>
</tr>
<tr>
<td>Grouper – RF2-5’</td>
<td>5’ - GAGCTAACGATGACGACAG</td>
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<tr>
<td>Grouper – RF2-3’</td>
<td>5’ - TCAATACATGCCCATTCAG</td>
</tr>
<tr>
<td>Grouper – RF7-5’</td>
<td>5’ - GTGACGCTGGTITTATATGTC</td>
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<tr>
<td>Grouper – RF7-3’</td>
<td>5’ - CAATCCACACAAACATGGA</td>
</tr>
<tr>
<td>Grouper – NACH-5’</td>
<td>5’ - AGCTGTCTTCTACGGC</td>
</tr>
<tr>
<td>Grouper – NACH-3’</td>
<td>5’ - CTAGCTCTACAGTCTCCAGTTA</td>
</tr>
</tbody>
</table>

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The control group was infected with V. vulnificus (strain 204; at 1.23 × 10^7 cfu/ml) without prior electroporation. A piece of muscle from the same region as the DNA injection site was removed from the fish at 3, 6, 12, 24, and 48 h after injection with 20 μl of V. vulnificus or electroporation alone. The muscle was soaked in culture buffer (TSB buffer + 2% NaCl) for 60 min at 37 °C, and then macerated using clippers. The culture buffer was serially diluted, and transferred to TSB plates for incubation. Bacterial colonies were subsequently counted to quantify the antimicrobial effect.

The expression of grouper immune-related genes was determined by extracting RNA from liver or muscle at the indicated times after each treatment, and performing qRT-PCR analysis as previously described [10, 23]. Primers were designed to amplify transcripts encoding tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and others.
NF-κB, MYD88, IL-8, TNFα, IRF1, IRF2, IRF7, MX, NACHT, and β-actin. Primer sequences are given in Table 1.

2.5. Statistical analysis

Data from each experiment were expressed as egfp (or immune-related gene) expression/β-actin expression. T-test was used to compare two groups. Multiple group comparisons were performed using analysis of variance (ANOVA) (SPSS software). Differences were defined as significant at (*) \(0.01 \leq p < 0.05\), (**) \(0.001 \leq p < 0.01\), (***) \(p < 0.001\). Different letters between groups indicate significant differences, while shared letters indicate no difference. All samples were examined in triplicate. Values represent the mean \(\pm\) SD of \(n\) experiments, where \(n\) represents the number of separate experiments for each RNA source.

3. Results

3.1. Optimal conditions for plasmid electroporation into grouper muscle

First, the optimal conditions for electroporation of pCMV-gfp-epi into grouper muscle were determined. GFP expression in muscles electroporated with pCMV-gfp-epi or PBS (control) was examined using qRT-PCR and fluorescent microscopy. The highest levels of GFP mRNA (relative to β-actin mRNA) were observed...
following electroporation using a voltage of 50 V (Fig. 1a), a pulse number of 2 (Fig. 1b), a time of 48 h (Fig. 1c), and a concentration of 70 μg plasmid DNA/fish (Fig. 1d).

3.2. Detection of CMV-gfp-epi and its transcripts in grouper tissues

To study the effect of electroporation of plasmid DNA into grouper muscle, qRT-PCR was used to analyze the levels of egfp mRNA in various organs (heart (Ht), gill (Gi), liver (Lv), kidney (Kd), spleen (Sp), muscle (Mu), and intestine (In)). Expression of egfp was significantly greater in muscle than in other tissues (Fig. 2a). To determine whether the circulatory system carried plasmid DNA to other tissues after electroporation, PCR was performed with primers against the pCMV-gfp-epi plasmid using genomic DNA from the tissues listed above; plasmid was detected in grouper muscle, heart, and liver (Fig. 2b).

3.3. Effect of electroporation of pCMV-gfp-epi on V. vulnificus infection

The effect of prior electroporation of grouper muscle with pCMV-gfp-epi on V. vulnificus infection was subsequently investigated. At 48 h after electroporation with pCMV-gfp-epi or CMV-gfp, grouper were infected with V. vulnificus. Muscle samples for analysis were collected at 3, 6, 12, 24, and 48 h after infection, as shown in Fig. 3a. Grouper muscle subjected to electroporation with either pCMV-gfp-epi or CMV-gfp exhibited an increase in bacterial cells from 24 h after infection; bacterial counts were increased further by 48 h (Fig. 3b). However, prior electroporation with pCMV-gfp-epi resulted in a significant inhibition of bacterial growth, as compared to electroporation with CMV-gfp.

To further delineate the effects of epi on the cellular response to V. vulnificus infection, the effect of pCMV-gfp-epi electroporation and subsequent infection on the expression of immune-related genes (IL-1β, NF-κB, TNF-α, MYD88, IL-8, TNF2, IRF1, IRF2, IRF7, MX, and NACHT) were analyzed in muscle (Fig. 4) and liver (Fig. 5). As compared to the CMV-gfp control, over-expression of epi (pCMV-gfp-epi) resulted in enhanced expression of the following immune-related genes in muscle: IL-1β (6 and 24 h), NF-κB (3, 6, and 24 h), TNF-α (3, 6, and 12 h), MYD88 (6, 24, and 48 h), IL-8 (6, 24, and 48 h), TNF2 (3, 6, and 24 h), IRF1 (3 h), IRF2 (3, 6, and 24 h), IRF7 (3, 6, and 24 h), MX (6 and 24 h), and NACHT (24 and 48 h) (Fig. 4, Supplementary Table 1). In the liver, expression of the following genes was increased by electroporation with pCMV-gfp-epi: IL-1β (6 and 24 h), NF-κB (3, 6, and 12 h), MYD88 (6, 24, and 48 h), IL-8 (6, 24, and 48 h), TNF-α (3, 6, and 24 h), TNF2 (3, 6, and 24 h), IRF1 (3 h), IRF2 (3, 6, and 24 h), IRF7 (3, 6, and 24 h), MX (6 and 24 h), and NACHT (24 and 48 h) (Fig. 5, Supplementary Table 1).
epi: IL-1β (3 and 48 h), NF-κB (3 h), TNF-α (6 and 24 h), MYD88 (3 and 48 h), IL-8 (3 and 48 h), IRF1 (3 h), IRF2 (24 h), IRF7 (3 h), and MX (3 h) (Fig. 5, Supplementary Table 1).

Electroporation of grouper muscle with pCMV-gfp-epi also affected the expression of certain immune-related genes in the absence of bacterial infection; IL-1β was significantly increased in muscle (Supplementary Fig. 2a), and IL-8, TNF-α, and IRF2 were significantly increased in liver (Supplementary Fig. 2b), as compared to both untreated grouper and grouper electroporated with CMV-gfp.

3.4. Differential effects of pCMV-gfp-epi electroporation and V. vulnificus infection on the expression of immune-related genes

Finally, the effects of pCMV-gfp-epi electroporation in muscle (Epi) on immune-related gene expression were compared with those of V. vulnificus infection (Vibrio). Samples were taken at various time points after electroporation or infection, as shown in Supplementary Fig. 3a. Two control groups were also prepared: (i) grouper injected with PBS instead of V. vulnificus (PBS), and (ii) grouper electroporated with pCMV-gfp instead of pCMV-gfp-epi (Vec). Bacterial numbers were confirmed to be increased only by V. vulnificus infection (Supplementary Fig. 3b). The muscle of grouper infected with V. vulnificus also exhibited significantly enhanced expression of IL-1β (3, 6, 12, 24, and 48 h), NF-κB (3, 6, 12, 24, and 48 h), IL-8 (3, 6, 12, 24, and 48 h), IRF1 (6 and 12 h), IRF2 (6 and 12 h), and IRF7 (12 h) (Fig. 6). On the other hand, overexpression of epi resulted in significant induction of IRF2 (0 and 3 h), IRF7 (0 h), MYD88 (0, 3, 24, and 48 h), MX (0 h), TNF2 (0, 3, 12, and 48 h), NACHT (0, 3, and 12 h), and TNF-α (3, and 6 h) (Fig. 6).
4. Discussion

The present study demonstrates for the first time that electrotransfer of plasmid DNA encoding epinecidin-1 (epi) reduces the severity of *V. vulni* [1]*cus* infection, and modulates expression of inflammatory genes in a marine fish, grouper. Our study also reveals that electrotransfer of epi DNA enhances expression of MYD88, TNFα, TNF2, NACHT, and IRF2 (at varying times) in the absence of *V. vulni* [1]*cus* infection.

The electroporation efficiency and potential damage inflicted by this method in marine fish remain unclear. Differences in the strength and specificity of the expression of electroporated genes between freshwater and marine fish may be partially attributable to the plasmid promoters. The cytomegalovirus (CMV) promoter resulted in greater epi activity than the mylz promoter in zebrafish muscle [10]. The bacterial clearance rate in zebrafish was also enhanced by placing epi under the control of this promoter [10]. However, electroporation of epi-encoding plasmids into grouper resulted in a decreased rate of bacterial clearance, as compared to zebrafish. This may be a consequence of reduced expression of the pCMV-gfp-epi plasmid in grouper, which may in turn result from inefficient delivery of DNA to grouper muscle cells; these cells are present in the basal layer of the epidermis [24,25]. The CMV promoter results in expression in all tissues, including the epidermis and dermis. Delivery of plasmid DNA to grouper muscle cells may be less efficient because of the increased depth of penetration necessary. Moreover, the expression patterns of the CMV promoter in grouper skin may be different from the patterns observed in zebrafish, pig, or human skin [10,26]. The electroporation of marine fish with plasmid DNA encoding an AMP has not previously been reported. The current finding that electroporation of epi...
plasmid DNA decreased bacterial growth in grouper muscle has promise, but further refinement of the technique is required to fully develop its potential.

Injection of fish with reporter plasmid has been reported to result in the persistence of protein expression from less than 7 days to more than 115 days, depending on the reporter gene and fish model used [31]. Muscle injection or gene gun bombardment of zebrafish were both reported to result in GFP expression for 50 days [29], while gene expression persisted for 535 days after injection of 100 μg of plasmid DNA into salmon muscle [27]. The results of this study confirm that injection of DNA followed by electroporation increases gene expression, as compared to injection alone. The present study demonstrates that in vivo electroporation of 70 μg of DNA is sufficient to exert bactericidal effects in grouper, and an earlier study reported that electroporation of dechorionated zebrafish eggs resulted in expression in...
nearly 25% of fish [28]. Electroporation has been shown to be a mild process, which is well-tolerated by organisms [30]. Furthermore, plasmid DNA injected into grouper muscle appears to persist (at least in part) in an extrachromosomal circular form, as observed in other models. Taken together, the present findings suggest that injection and electroporation of grouper muscle with foreign DNA results in persistent expression, which is spatially restricted.

Earlier studies have investigated the effect of introducing plasmids encoding immune system components on the immune response: plasmids encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) elicited CD8+ T-cell responses in melanoma patients [32], and plasmids encoding IL-2 increased humoral and cellular responses in animal models of infection with Mycobacterium tuberculosis, bronchitis virus, or papillomavirus [27,33–35]. The present study examined the effect of epi plasmid electroporation and V. vulnificus infection on the expression of several immune-related genes in grouper muscle. The results were similar with previous findings in zebrafish; prior electroporation with pCMV-sgp-epi significantly decreased expression of IL-1β and TNF upon bacterial infection, as compared to controls [10]. Although the underlying mechanisms remain unclear, the slow rise of the immune response following DNA immunization suggests a complex process that may mimic the natural function of AMP, in a similar manner to DNA vaccines [36]. Following electroporation, the muscle cells translate epi into protein, which may then be presented by MHC-I to the immune system. Epi may trigger multiple arms of the immune response, thus providing flexibility in immune system modulation.

This study also examined the effect of the electroporation of the epi gene in the absence of bacterial infection; increases in the levels of transcripts encoding MYD88, TNF-α, TNF2, and NACHT were observed at different times following electroporation. These results are similar to earlier reports that MyD88 signaling in CD4 T cells promotes IFN-γ production in response to intracellular bacterial infection [37]. Microarray and quantitative PCR analysis have also revealed that transcription factors, such as NF-κB and AP-1, play an important role in innate immunity, and the pro-inflammatory cytokine IL-1β is dependent on MyD88 signaling during bacterial infections [38]. Taken together, the current results suggest that epi may provoke an inflammatory response that elevates the expression of immune-related genes in response to bacterial infection.

In conclusion, this study has demonstrated that epi plasmid DNA induces up-regulation of several immune-related genes, especially those relating to innate immunity, in grouper (E. coioides). Further elucidation of the mechanism underlying the anti-bacterial effect of electroporated epi DNA is required for validating its safety for use in aquaculture.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2013.07.050.

References


