Feeding the transgenic microalgae containing bovine lactoferricin enhances the survival of fish infected by *Vibrio parahenmolyticus*

Si-Shen Li and Huai-Jen Tsai

Institute of Molecular and Cellular Biology, National Taiwan University, Taipei, Taiwan

Key words: *Nannochloropsis oculata*, lactoferricin, *Vibrio parahenmolyticus*, microalgae.
Abstract:

Microalga, *Nannochloropsis oculata*, is an important microorganism for feeding fish larvae. We develop a transgenic line of *N. oculata* that enables to produce an antimicrobial peptide, bovine lactoferricin (LFB). An algae-codon-optimized cDNA of LFB was fused with a red fluorescent protein (DsRed) reporter and driven by a heat-inducible promoter, which is a heat shock protein 70A promoter combined with a ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit 2 promoter from *Chlamydomonas reinhardtii*. This construct was transferred into *N. oculata* cells by electroporation. The successful rate of stable transgenesis at least for 15 months was around 0.4% (2/491) of the examined microalgae clones. After the heat induction, the transcripts of LFB-DsRed gene was detected in the transgenic algal clone (0829A) by RT-PCR. In addition, protein analysis showed that a band corresponding to the fusion protein of LFB-DsRed was positive in the immunoblotting analysis by using monoclonal anti-DsRed antibody. When we orally-in-tube fed injected with algae into medaka, then infected by *Vibrio parahaemolyticus* for 6 hr, we calculated the survival rate after 24 hr infection. Results showed that the survival rate of medaka fed with recombinant algae (1×10^8 cells/per fish) was greatly higher than that of medaka fed with wild-type algae, 91.6% (n=12) versus 0% (n=12), suggesting that the transgenic microalgae, 0829A, can help to enhance the survival of medaka after *V. parahaemolyticus* infection.
Introduction

The aquaculture’s products are one of important food resources for humans. To meet the requirement of increasing population, the large-scale and high-density aquaculture systems are developed intensively. But the yield of aquaculture may be declined markedly if fish and shellfish diseases occur. Under this circumstance, chemical antibiotics are commonly employed in the most economic fish culture to prevent the aquaculture’s products from being suffered diseases, except that only large-sized and expensively fish like trout and salmon can be inoculated with vaccine. Subsequently, the antibiotic resistant pathogens may be selected and spread out along with the waste water to cause seriously environmental pollution. Therefore, to develop a safety, effective and inexpensive biological antibiotic for aquaculture is extremely necessary.

Bovine lactoferricin (LFB) is a 3,142-kDa peptide derived from the N-terminus of bovine lactoferrin (from Phe17 to Phe41). LFB can be generated by pepsin hydrolysis in the digestive tract (Bellamy et al., 1992a). LFB is an antimicrobial peptide that enables to kill or restrain many pathogens, including Gram-negative bacteria, such as *Escherichia coli* (Bellamy *et al.*, 1992a), *Proteus vulgaris* (Bellamy *et al.*, 1992b), *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Yamauchi *et al.*, 1993); Gram-positive bacteria, such as *Clostridium paraputrificum*, *Corynebacterium ammoniagenes*, *Enterococcus faccalis* (Bellamy *et al.*, 1993c), *Listeria*
monocytogenes (Wakabayashi et al., 1992) and Streptococcus bovis (Bellamy et al., 1992b); parasites, such as Eimeria stiedai (Omata et al., 2001), Giardia lamblia (Turchany et al., 1995), Toxoplasma gondii (Tanaka et al., 1995); fungi, such as Aspergillus fumigatus, Penicillium pinophilum (Bellamy et al., 1994); and virus, such as adenovirus (Biase et al., 2003), calicivirus (McCann et al., 2003) and cytomegalovirus (Andersen et al., 2001). Since LFB can suppress general kinds of pathogens, LFB is an excellent material for applying on aquaculture. However, LFB is hardly accessible: it is produced only by the hydrolysis of bovine lactoferrin under pepsin treatment in native source (Tomita et al., 2002). This limitation can be solved by making LFB be produce by microalgae.

Microalgae are potential materials for using as bioreactors to produce the heterologous proteins mainly because microalgae have many advantages: (1) microalgae enable to produce complicate eukaryotic proteins after post-translational modification (Mayfield et al., 2003); (2) although microalgae are eukaryotic, they can be cultured as cheap and fast as bacteria; and (3) microalgae are considered as food safe microorganisms due to they are free from human pathogens and endotoxin (Tara et al., 2005). Nannochloropsis oculata is a marine unicellular microalga, belonging to Class of Eustigmatophyceae, with a spherical or slightly ovoid shape about 2 to 4 mm in diameter. It consists of polysaccharide wall and contains only one chloroplast
(Hibbered, 1981). Because *N. oculata* can grow in wide range of saline concentration (Hirata *et al.*, 1980) and contains high amount of eicosapentaenoic acid (Sukenik *et al.*, 1993), it is an important microorganism for feeding fish larva and for making green-water of aquaculture ponds (Fulks *et al.*, 1991; Lubzens *et al.*, 1995). In addition of the above advantages that general microalgae have, *N. oculata* can be cultured in an extremely huge scale by using seashore and sunny sea pond only with fertilizer without constraining the limitations of the shortages of freshwater and cultivable land. Unlike yeast and cell line cultures, expensive medium and aseptic manipulation of *N. oculata* are not required. More over, *N. oculata* can survival in widespread salinity, so that it can be applied both in freshwater aquaculture and in seawater aquaculture. The feeding experiments also demonstrate that *N. oculata* enables to reduce blood pressure in hypertensive rats (Seto *et al.*, 1992). Importantly, *N. oculata* does not generate gamete (Maruyama *et al.*, 1986). Gamete-based contamination of genetically modified organism has not to be concerned. Lastly, transgenic *N. oculata* is already available due to cryopreservation technique for *N. oculata* has been well developed (Poncen and Veron 2003, Cwo *et al.*, 2005).

Many studies have been reported on microalgal gene transfer (Boynton *et al.*, 1988; Brown *et al.*, 1991; Kumar *et al.*, 2004; Geng *et al.*, 2003; Borovsky 2003; Geng *et al.*, 2003; Mayfield *et al.*, 2003; Sun *et al.*, 2003; Banicki 2004). However, the
selection marker all they used to screen the transformants was antimicrobial resistant gene. Using antimicrobial resistant gene to serve as a bioreactor has to concern about the biological safety, especially if the transgenic microalgae are largely cultured in an open area. In this study, we develop an antimicrobial-free gene transfer system for *N. oculata* by using red fluorescent protein (DsRed) gene, which originates from coral (*Discosoma sp*), as a selective marker. In order to release the LBF from the LFB-DsRed fusion protein produced by transgenic *N. oculata*, we reserved two pepsin cleave sites at Phe2-Lys and at Phe26-Met of LFB-DsRed. It made LFB can be released from LFB-DsRed in aquatic organism’ digestive tract by pepsin digestion. On the other hand, the expression of this fusion protein avoids the destroy of bioreactor when LFB were express along (Kim *et al.*, 2006).

We created the transgenic *N. oculata* to expresses this heterogeous LFB-DsRed fusion protein driven by an inducible promoter from *Chlamydomonas reinhardtii* (Schroda *et al.*, 2000): a heat shock protein 70A promoter (*HSP 70A*) combined with ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit 2 ‘ promoter (*RBCS 2*). The detection of Ds-Red monoclonal antibody and fluorescent microscope demonstrate that the transgenic *N. oculata* can express LFB-DsRed. After bioassay, the medaka that naturally living in fresh water were domesticate in to sea water orally-in-tube fed injected with tansgenic microalgae, then infected by *Vibrio*
parahaemolyticus for 6 hr. the survival rate showed that the medaka fed with recombinant algae ($1 \times 10^8$ cells/ per fish) was greatly higher than that of medaka fed with wild-type algae, 91.6% (n=12) versus 0% (n=12), suggesting that the transgenic microalgae, 0829A, can help to enhance the survival of medaka after V. parahaemolyticus infection.
Materials and methods

Plasmid construction and preparation

An inducible promoter of algae expression, $Hsp70$ A plus $RBCS$ 2, was obtained by cutting with $Nhe$ I and $EcoR$ V from pCB740 (Schroda et al., 2000). A cDNA of fusion protein, an algae-codon-optimized (Naya et al., 2001) of LFB fused with a DsRed reporter, driven by this inducible promoter was generated by the following sequential PCR. Plasmid pDs-Red 2-1 (Clonetech), containing the DsRed cDNA of $Discosoma$ sp., was used as a template. Four constructed primers (CF3, CF2, CF1 and CR) were synthesized: contained the $Nhe$ I cutting site, cDNA of algae-codon-optimized LFB and partial cDNA of N terminus (10 bp) of DsRed. And one reverses primer, CR, which contained partial cDNA of C terminus of DsRed and the $EcoR$ V cutting site. Primers of CF1 and CR were used in the first PCR reaction under the following condition: 5 cycles of denaturing at 94 °C for 30 sec, annealing at 30 °C for 15 sec and extension at 72 °C for 90 sec, followed by 20 cycles of denaturing at 94 °C for 30 sec, annealing at 60 °C for 15 sec and extension at 72 °C for 90 sec. The resultant 734-bp-PCR product was extracted from a 2 % agarose gel after electrophoresis. This PCR product was used as the template DNA for the second PCR reaction using primers of CF2 and CR. The resultant 763-bp-PCR product was used the template for the third PCR reaction by using primers of CF3 and CR. The final
802-bp-PCR product was cloned into plasmid pGEM-T-easy. After the resultant plasmid was digested with Nhe I and EcoR V, the fragment containing an inducible promoter (hsp70A plus rbcs2) was inserted to pCB740 which digested by same restriction enzyme set to generate the algal LFB expression plasmid, phr-rLFB-Red. This plasmid was linearized by Sac II to be ready for using on gene transfer.

**Culture conditions and protoplast preparation of microalgae**

*N. oculata* obtained from Taiwan Fisheries Research Institute were cultured in f/2 medium (Guillard 1975) at 28°C with an illumination of white fluorescent tubes (Taiwan fluorescence company: FL40D/38) on a 12:12 h light/day cycle. For preparing protoplasts, 5 ml of *N. oculata* cells (1 x 10^7 cell/ml) were cultured for 5-6 days and collected by centrifugation at 8,000 X g for 10 min at 4°C. The pellet was washed twice with 1ml of sterilized distilled seawater, resuspended in a 500µl of synthetic gastric juice (Atkins *et al.*, 1998), and incubated at 37°C in the dark with gently shaking. After treatment, cells were washed twice with 1ml of sterilized distilled seawater to terminate enzymatic activity. Finally, the pellet was resuspended with 0.2 ml electroporation buffer (Chen *et al.*, 2001) and chilled on ice for 10 min for ready for electroporation.

**Gene transfer by electroporation**

One half (0.1ml) of *N. oculata* protoplasts resuspended in the electroporation
buffer was added with 10µg of SacII-restricted phr-rLFB-Red. Then, the gene transfer was performed by electroporator (ECM 2001, BTX, U.S.A.) to generate the transgenic *N. oculata*.

**Regeneration and growth**

After electroporation, the *N. oculata* was transferred to a glass tube containing 1 ml of fresh f/2 medium and cultured at 28°C for 3-5 days with agitation at 200 rpm. Twenty microliters of algal cells grown at log-phase (1×10^7 cells/ml) were spread on an agar plate containing f/2 medium and incubated at 28°C for 5-7 days. Each colony was picked up and cultured in liquid medium as described above for subsequent analysis.

**Genomic DNA extraction**

The genomic DNA extraction of *N. oculata* was followed the description of Dawson *et al.*, (1997) with some modifications. Microagal cells were harvested from 5 ml (approximately 1 x 10^7 cells/ml) of culture medium and resuspended in 500 µl of buffer solution (54 mM hexadecyltrimethylammonium bromide, 0.25 mM Tris, pH 8.0, 1.4 M NaCl, 10 mM EDTA, and 2% β-mercaptoethanol). The mixture was incubated at 65°C for 2 h and shaken every 15 min. After incubation, an equal volume of phenol–chloroform was added and the aqueous phase was recovered after 5 min of centrifugation at 8,000 X g at 25℃ for 10 min. We extracted several times until the
aqueous layer was no longer cloudy. The genomic DNA was precipitated with 2 volumes of 100% ethanol, centrifuged at 8000 X g for 15 min, washed with 70% ethanol, dried, and resuspended in 30 µl of TE buffer.

**PCR analysis**

Two oligonucleotide primers were synthesized for detection of the existence of the transferred DNA fragment, Ds-Red cDNA, by PCR analysis: a forward primer (5’-CCTCCTCCGAGAACGTCATCACCGAG-3’) and a reverse primer (5’-CCTCGGTGCGCTCGTACTGCT-3’). The primers for detection of the endogenous 18S rRNA gene, which served as an internal control, were forward primer (5’-GCGGAGGAAAAG-AACTAACCAGGATT-3’) and reverse primer (5’-AACGCCATGGCACAACC-GC-3’). Each PCR sample consisted of 20 µl of solution containing 10-20 ng of template, 10 pmol of each primer, 25 µM of each dNTP, and 5 units of taq enzyme (Viotech, Taiwan), in a 10× PCR buffer. Amplification was performed with a Perkin-Elmer Cetus DNA Thermal Cycler (Maryland, U.S.A.). PCR consisted of 25 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, followed by 10 min extension at 72°C. PCR products (10 µl) were subjected to electrophoresis on a 3% NuSieve GTG agarose gel (FMC BioProducts, U.S.A.).
Preparation of microalgae’s mRNA

After 40 ml of wild-type (W) and transgenic (T) microalgae (1x10^7 cell/ml) were treated at 42°C for 16hr, Microagal cells were harvested from culture medium and resuspended in 1 ml of REzol (PROtech U.S.A.). The mixture was shaken for 30 sec and incubated at 25°C for 5 min. After incubation, 200 ul chloroform was added and the aqueous phase was recovered after 5 min of centrifugation at 8,000 X g at 4°C for 10 min. The genomic DNA was precipitated with 0.6 volumes of isopropanol, and incubated for 2 hr then centrifuged at 8000 X g for 15 min at 4°C, washed with 70% ethanol, dried, and resuspended in 30 µl of DEPC water.

Reverse transcription-polymerase chain reaction (RT-PCR)

First-strand cDNA was synthesized using the SuperScript II Pre-amplification System (Gibco BRL). Primer LF & LR were used to amplify a 68-bp DNA fragment from the transcript of recombinant bovine lactoferrin (rLFB) cDNA, whereas primer PF & LR were used to amplify a 380-bp PCR fragment from the contaminated plasmid DNA.

Induction, Protein extraction, and Western blot analysis.

Fifty milliliters (about 1 x 10^7 cells/ml) of the transformed *N. oculata* were heat shocked by shifting to 42°C for protein expression for 16hr, protein extracted from the transgenic *N. oculata* was modified using the protocol of Hawkins and Nakamura
Briefly, after microalgae were collected by centrifuging for 10 min at 8,000 X g in 4°C, the pellet was resuspended in 500 µl of extraction buffer (Franklin et al., 2002). The cell suspension was transferred to a microtube (Axygen, U.S.A.) containing 0.5-g glass beads (Sigma, U.S.A.), and the cells were broken by a mini-beadbeater (Biospec products, U.S.A.). Each session of breaking cells was at room temperature for 30 sec, and then the microtubes were put on ice for 90 sec. We repeated this procedure at least 15 times. The supernatant was collected by centrifuging for 15 min at 10,000 X g at 4°C, and 50 µl of sample loading buffer (1 mM EDTA, 250 mM Tris-HCl (pH 6.8), 4% SDS, 2% β-mercaptoethanol, 0.2% bromophenol blue, 50% glycerol) was added. Prior to SDS-PAGE analysis, samples were boiled for 40 min and then centrifuged for 5 min at 10,000 X g. The supernatants were electrophoresed on a 12% SDS–polyacrylamide gel. A mouse monoclonal antibody against DsRed (Clonetech, U.S.A.) was used to detect the fusion protein of LFB-DsRed. The final dilution of monoclonal antibody was 1:2000, and alkaline phosphatase-conjugated anti-rabbit IgG (Santa Cruz, U.S.A.) were used as the secondary antibody. The procedures for Western blot analysis were described previously (Tsai et al., 1993).

**Biological activity of LFB produced by N. oculata**

Medaka was domestication from fresh water to seawater in five days. Orally
injected wild type and recombinant algae into medaka in with different concentration (1×10^8 or 1×10^6 cells/ per fish) after shifting to 42°C for protein expression for 16hr, then infected by *Vibrio parahaemolyticus* for 6 hr, after 24 hr infection the survival rate were calculated.
Results and discussion

Preparation of *N. oculata* transformants with synthetic gastric juice treatment and electroporation.

After *N. oculata* were cultured at log-phase to a density of $1 \times 10^7$ cells, we treated them with synthetic gastric juice (Atkins *et al.*, 1998), and incubated at 37°C in the dark with gently shaking. The algae treated with synthetic gastric juice get 1248 clones after electoration.

Screening the putative transgenic clones of *N. oculata* by PCR analysis

After treatment of electroporation, we used PCR analysis to screen for genomic DNA extracted from *N. oculata* at the third generation. We screened 419 transformants grown on the cultured plates, a single 450-bp PCR product was found (Figure 2). This positive band corresponded to that amplified from the DNA fragment used for gene transfer. More than 50 % clones were transformants. However, after the 15 months, we found PCR-positive signal in only 2 transgenic clones among 419 clones examined

Because antibiotics did not affect the growth of microalgae, *N. oculata* survived in the medium whether the cells contained the plasmid phr-rLFB-Red or not. We used PCR to examine each colony whether they contained the transferred gene fragment or not. Only two clones (0829A and 0829B) of the microalga were obtained after
subculture for 15 months.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

RT-PCR analysis of mRNA extracted from 20 ml microalgae (1x10^7 cell/ml) after they were induced at 42°C for 16hr. Primer LF & LR were used to amplify a 68-bp DNA fragment from the transcript of recombinant bovine lactoferricin (rLFB) cDNA, whereas primer PF & LR were used to amplify a 380-bp PCR fragment from the contaminated plasmid DNA (Fig 3A). Three transgenic microalgae which had the positive signal indicate that phr-rLFB-Red can produced the mRNA of fusion protein gene which contain the open-reading-frame of rLFB and DsRed (Fig 3B).

**Protein and Western blot analysis.**

The result of SDS polyacrylamid gel electrophoresis indicated that the transgenic microalgae (0829A) can express the tetramer of recombinant fusion protein after treated at 42°C for 16hr. In non-induction transgenic algae, a few of recombinant fusion protein also be express. This miss-expression also observed by same expression system in microalgae (Schroda et al., 2000)

**Biological activity of LFB produced by *N. oculata***

we orally-in-tube fed injected with algae into medaka, then infected by *Vibrio parahaemolyticus* for 6 hr, we calculated the survival rate after 24 hr infection. Results showed that the surivial rate of medaka fed with recombinant algae (1x10^8 cells/ per fish) was greatly higher than that of medaka fed with wild-type algae,
91.6% versus 0% (n=12), suggesting that the transgenic microalgae, 0829A, can help to enhance the survival of medaka after *V. parahaemolyticus* infection.
Figure 1.
Figure 2
Figure 4.
Table 1. The survival rate of medaka with alga injection and *V. parahaemolyticus* infection

<table>
<thead>
<tr>
<th>Injection</th>
<th>Dsw</th>
<th>Wild type</th>
<th>TL</th>
<th>TH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dsw</td>
<td>100 %</td>
<td>100 %</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td>Cont</td>
<td>100 %</td>
<td>100 %</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td>VP-L</td>
<td>0 %</td>
<td>0 %</td>
<td>81.8%</td>
<td>100 %</td>
</tr>
<tr>
<td>VP-H</td>
<td>0 %</td>
<td>0 %</td>
<td>66.7%</td>
<td>91.6%</td>
</tr>
</tbody>
</table>

(N= 12)
Figure 1. Expression vector (phr-rLFB-Red) for microalgae to produce recombinant bovine lactoferricin. *Hsp70A* + *RBCS 2*: a promoter of heat shock protein 70A gene fused with a promoter of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit 2 gene of *Chlamydomonas reinhardtii*; rLFB : recombinant bovine lactoferricin but had been algae-codon-optimized. Open arrows indicate the sites where is pepsin to release the LFB; DsRed: red fluorescent protein gene of *Discosoma sp* (Clontech). *Nhe* I and *EcoR V* are two restriction enzyme cutting sites for inserting the DNA fragment which encodes the fusion protein (rLFB with DsRed) into the pCB740 vector. DF and DR are the primers used for screening of transformants to amplify a 450-bp PCR products of phr-rLFB-Red.
Figure 2. PCR detection of the transformed microalgae. Microalgae were electroporated with 10µg/ml linear form plasmid (phr-rLFB-Red). After the 3rd generation, 1 ml microalgae (1x 10^7 cells) were treated with 50µg DNase I at 37°C for 1 hr and inactivated at 65°C for 20 min before their genomic DNA were analyzed by PCR. Lane M: molecular marker, N: negative control (no DNA template); P: positive control (1 ng of linear plasmid: phr-rLfcB-Red), 1~11: single colony of microalgae, W: wild-type alga that was incubated with 10µg/ml linear plasmid but without electroporation. When primers of DF and DR were used, a 450-bp PCR product was expected to be produced by transgenic microalgae. Amplification of 18srRNA was served as an internal control.
Figure 3. RT-PCR analysis of microalgae. A: transgene and the primers designed for detecting the transgene (PF and LR) and its mRNA (LF and LR). Primer LF & LR were used to amplify a 68-bp DNA fragment from the transcript of recombinant bovine lactoferricin (rLFB) cDNA, whereas primer PF & LR were used to amplify a 380-bp PCR fragment from the contaminated plasmid DNA. B: RT-PCR analysis of mRNA extracted from 20 ml microalgae (1x10^7 cell/ml) after they were induced at 42℃ for 16hr. M: molecular marker, P: positive control (1 ng of phr-rLFB-Red), N: negative control (no DNA template), W: wild-type microalga, lanes 1~4: transgenic microalgae, while 18s rRNA was served as an internal control.
Figure 4. After 50 ml of wild-type (W) and transgenic (T) microalgae (1x10^7 cell/ml) were treated at 42 °C for 16 hr, their total proteins were extracted, and analyzed by 12 % SDS polyacrylamid gel electrophoresis (A). And transferred into nitrocellulose membrane and analyzed by monoclonal anti-Ds Red antibody (B). W: wild type microalgae, T: transgenic microalgae. Arrow indicated the tetramer of recombinant fusion protein produced by transgenic microalga.
Table 1. Medaka were first injected with transgenic *N. oculata* then infected by *V. parahaemolyticus* after 6 hr, and calculated the survival rate after 24 hr *V. parahaemolyticus* infection. dSW: 10 µl distill sea water; Cont: dead *V. parahaemolyticus* (1x10^5 cells/ per fish) which boiled for 30 min; VP-L: Living *V. parahaemolyticus* (1x10^4 cells/ per fish); VP-H: Living *V. parahaemolyticus* (1x10^5 cells/ per fish); Wild type: wild type *N. oculata* (1х10^8 cells/ per fish); TL: transgenic *N. oculata* (1 х10^6 cells/ per fish); TH: transgenic *N. oculata* (1 х10^8 cells/ per fish). N=12.
References


Karlson, B., D. Potter, M. Kuylenstierna, and R. A. Andersen. 1996. Ultrastructure, pigment composition, and 18S rRNA gene sequence for Nannochloropsis granulata sp. nov. (Monodopsidaceae, Eustigmatophyceae), a marine ultraplankter isolated from the


