Construction of Recombinant *Vibrio harveyi* to Study its Adherence in Shrimp Larvae

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ABSTRACT

Vibrio harveyi M, G_3, and G_7 are shrimp-larvae pathogens which were isolated from different locations in Indonesia. Each of the three isolates demonstrated unique genotypes as shown by pulsed-field gel electrophoresis (PFGE). The gene for green fluorescent protein (*gfp*) was inserted into a broad-host range plasmid pBBR1MCS2 to generate a recombinant plasmid pWG01, which was introduced into the three isolates of *V. harveyi* employing triparental mating. *V. harveyi* isolates carrying pWG01 resulted in green-fluorescent colonies and cells due to the production of GFP. Growth curve analysis showed that each of the recombinant *V. harveyi* exhibited almost identical profiles to that of the wild type parental strain. Introduction of pWG01 into the three isolates did not alter their pathogenicity. However, after 48 h of incubation in antibiotic-free medium, approximately 50% of the cells had lost their recombinant plasmid carrying *gfp*. Expression of *gfp* in *V. harveyi* will be presented. In addition, the application of the recombinants for the study of adherence will be introduced.

INTRODUCTION

V. harveyi was identified as a causative agent of mass mortalities of shrimp larvae and were frequently associated with luminous *Vibrio* (Lavilla Pitogo *et al*., 1990; Karunasagar *et al*., 1994; Ruangpan, 1998). Luminous vibrios isolated from shrimp hatcheries on Java Island, Indonesia and from pristine marine habitats, have demonstrated multiantibiotic resistance to antimicrobials like ampicillin, tetracycline, amoxicillin, and streptomycin (Tjahjadi *et al*., 1994; Soewanto *et al*., 1998; Teo *et al*., 2000).

Virulence in *V. harveyi* associated with toxic extra-cellular protein and luminescence was co-regulated by an intercellular quorum sensing mechanism. Therefore, intercellular signalling antagonism has potential utility in the control of *V. harveyi* shrimp infections (Manefield *et al*., 2000). Furthermore, Verschuere *et al*. (2000) described that bacterial adhesion to tissue surface was important during the initial stages of pathogenic infection,

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so that competition for adhesion sites with pathogens might be a primary probiotic effect.

Pathogenicity assays based in Koch's Postulates (Salyer and Whitt, 1994) were practically difficult to be conducted in shrimp larvae due to their relatively small size and lack of availability of *Vibrio*-free larvae (Hameed, 1993; Widanarni and Suwanto, 2000). Investigations into adherence and pathogenicity processes of this disease might be greatly facilitate if a visible marker could be introduced into the bacterial cells.

One visible molecular marker which has extensively been used for studying bacterial activity in the environment is gfp, i.e. a gene encoding green fluorescent protein (GFP) from a jellyfish (*Aequorea victoria*) (Manning, 1997). As a molecular gene marker, GFP has some advantages, such as no requirement for exogenous substrate or energy source for their visualization, sensitivity of detection, high stability, lack of toxicity, and no disturbance in cell function and growth (Josenhans *et al*., 1998; Ling *et al*., 2000).

GFP as a molecular marker has been used to demonstrate the mechanism of *Edwardsiella tarda* infection on epithelial cells of giant gouramy (Ling *et al*., 2000); and *Pseudomonas plecoglosicida* infection in ayu (*Plecoglossus altivelis*) (Sukenda and Wakabayashi, 2001). GFP was also successfully used as a marker in lactic acid bacteria (*Lactobacillus plantarum* and *L. lactis*) to study the possibility of using the bacteria as live vaccine carriers (Geoffrey *et al*., 2000).

In this report, we describe the construction and expression of GFP-carrying plasmids in three *V. harveyi* strains that differed genotypically based on *Not*I-schizotyping. GFP-tagged *V. harveyi* from M strain was used in a preliminary study to examine the adherence and pathogenicity of *V. harveyi* in shrimp larvae.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study and their relevant characteristics are described in Table 1. *E. coli* and *V. harveyi* were grown in Luria Bertani (LB) medium at 37°C and Seawater Complete (SWC) medium at 28°C, respectively. LB medium was made as previously described (Sambrook *et al*., 1989), SWC medium contained 5 g bactopeptone, 1 g yeast extract, 3 ml glycerol, 15 g agar, 750 ml seawater, and 250 ml distilled water.

Plasmid construction and molecular techniques

Recombinant plasmid pWG01 which has the *lac* promoter was constructed and used to drive the expression of *gfp*. The promoter and GFP gene were isolated from pSKL01 using *Eco*RI sites and ligated into pBBR1MCS2 linearized with *Eco*RI. The recombinant plasmid vector was transformed into *E. coli* DH5(using a standard heat shock transformation (Sambrook *et al*., 1989) and the colonies carrying pWG01 were examined for green fluorescence under UV-trans-illuminator at 312 nm (Biometra Ti 1, Gottingen).

Plasmid extraction, restriction enzyme digestions, agarose gel electrophoresis, gel isolated DNA fragment purification, and ligation were carried out using standards methods (Sambrook *et al*., 1989), and following the appropriate manufacturer's instructions. Restriction endonucleases and other enzymes were obtained from New England Biolabs Inc.

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strains or plasmids	Relevant characteristic (s)	Source or reference
V.harveyi strains		
M	Wild type	Maros Lab. collection
G3	Wild type	Gondol Lab. collection
G7	Wild type	Gondol Lab. collection
E.coli strains		
DH5α	F^- *lac*ZΔM15 *rec AI*	Sambrook *et al*. (1989)
HB 101	hsdR17	Sambrook *et al*. (1989)
	Res^- Mod^- *rec A13* Sm^R	
Plasmids		Sukenda and Wakabayashi
pSKL01	Gm^R, P_{lac}, gfp^+	(2001)
PBBRIMCS2	Mob^+, rep^+, MCS from	Kovac *et al*. (1994)
pRK2013	pBluescript 11, Km^R	Ditta *et al*. (1980)
pWG01	Col E1 replicon, Tra^+ of	This work
	RK2, Km^+	
	Km^R, P_{lac}, gfp^+	

Bacterial mating

To transfer the recombinant plasmid-vector into *V. harveyi* we used tri-parental mating with *E. coli* HB101 (pRK 2013) as helper cells. *E. coli* DH5α (pWG01) donors and *E. coli* HB101 (pRK 2013) helpers were grown overnight in LB medium supplemented with kanamycin (Km) 50 µg/ml at 37°C; whereas *V. harveyi* recipients were grown in SWC medium at 28°C. Each 1.5 ml of the donor, helper and recipient were pelleted in a micro-centrifuge at maximum speed for 1 min, and then the cells were washed with 1.0 ml 0.85% NaCl, re-centrifuged, and suspended in 40 µl of LB medium before being spotted onto a filter (1 cm diameter; pore size 0.45 µm; Millipore) on LB medium plate. The bacteria were allowed to conjugate at 28°C for 16 to 18 hours. At the end of the mating period, the filter containing the bacterial mixture was transferred into 1.5 ml microfuge tube containing 0.8 ml of 0.85% NaCl. The bacterial cells were suspended thoroughly by agitation in a vortex mixer.

The trans-conjugates were selected on Thiosulphate Citrate Bile Salt (TCBS, Oxoid) medium supplemented with Km (50 µg /ml). The selective medium TCBS was used to inhibit the growth of *E. coli* while allowing *V. harveyi* transconjugants harboring pBBR1MCS2 and its derivatives (*gfp* DNA fragments cloned into the multiple cloning site of pBBR1MCS2 and resistant to kanamycin) to grow normally.

Fluorescence measurement

GFP-expressing cells were visualized under a fluorescence microscope (Olympus BH2-RFCA, Japan) fitted with GFP filter set to examine cells. The amount of fluorescence emitted by cultures were also assayed using RF-540 spectrophotometer (Shimadzu, Japan) set to excite the cells at 395 nm and detect emission at 509 nm. Cultures were grown in SWC broth with antibiotic overnight at 28°C. The cells were pelleted and suspended in the same volume of 10% SWC, and the fluorescence was measured.

Cell growth and plasmid stability

V. harveyi strains carrying pWG01 were grown overnight in SWC broth supplemented with kanamycin. The cells were diluted at 1:100 in SWC broth without antibiotic, and the cell growth was determined hourly at A_{660}. For the plasmid stability assay, sequential propagation under non selective conditions were performed by inoculating with 1:100 (v/v) to assess plasmid survival by comparing duplicate colony counts on selective and non selective plates.

Adherence and pathogenicity assays

Two groups of shrimp post-larvae (PL_4) were immersed for 30 min in 10^6 CFU/ml of pWG01 and wild type of *V. harveyi* (final concentration), respectively, and then placed in a 2 liter shrimp rearing tank for five days. A control group was immersed in SWC broth. Samples from dead shrimp larvae were inoculated on SWC plates with or without antibiotic and were observed under a fluorescence microscope.

RESULTS AND DISCUSSION

Plasmid construction

The GFP-plasmid vector was constructed to be used as a molecular marker in *V. harveyi*. The GFP vector, pWG01, was constructed with *lac* promoter to drive the expression of GFP (Fig. 1). When pWG01 was introduced into *V. harveyi* by triparental mating, fluorescent colonies could be easily identified on SWC plates by eye with UV-trans-illumination at 312 nm. Stretton *et al*. (1998) reported that among three strains of marine bacteria studied, *gfp* expressed from a *lac* promoter could be visualized in *Vibrio* sp. strain S141 but was not functional in either *Pseudoalteromonas sp*. strain S91 or *Psychrobacter* sp. strain SW511. The *gfp* also was not, or was poorly expressed, from a *lac* promoter in *Pseudomonas fluorescens* (Bloemberg *et al*., 1997) and *P. plecoglossicida* (Sukenda and Wakabayashi, 2001).

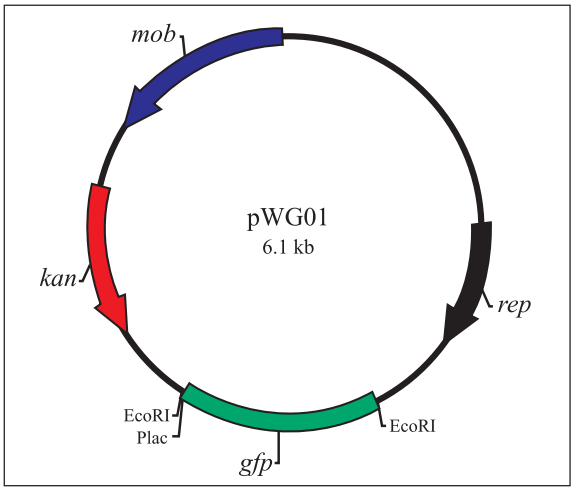

Figure 1. Structure of the *gfp* expression vector, constructed as described in materials and methods.

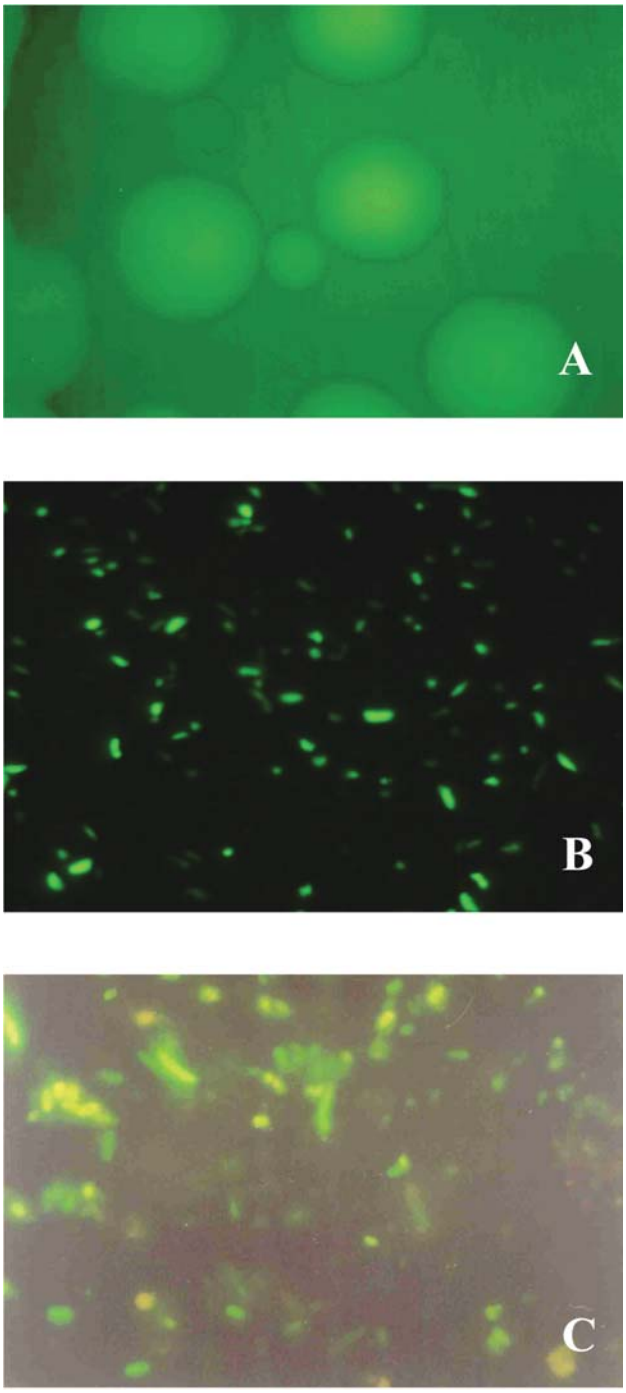

Figure 2. Fluorescence micrographs of recombinant *V. harveyi* carrying pWG01.
(A) Recombinant *V. harveyi* colonies;
(B) Recombinant *V. harveyi* cells;
(C) Recombinant *V. harveyi* adhering to the carcasses of shrimp larvae.

Expression of GFP in *V. harveyi*

Expression of GFP in *V. harveyi* was demonstrated by fluorescence microscopy of colonies and of individual cells (Fig. 2). When fluorescence was measured by fluorometry, the emission values were as follows: 167, 192, 193 for *V. harveyi* strains M, G3, G7 carrying pWG01 respectively. On the other hand emission values for wild type strains of M, G3, and G7 were 14, 12, and 13 respectively. Emission value of 171 and 16 fluorescence units were detected for *E. coli* DH5α (pWG01) and its wild type strain respectively. All *V. harveyi* strains and *E. coli* DH5α carrying pWG01 produced similar fluorescence levels and had lower intensities of green fluorescence compared with *E. coli* carrying pSKL01 that was emitting 980 fluorescent units.

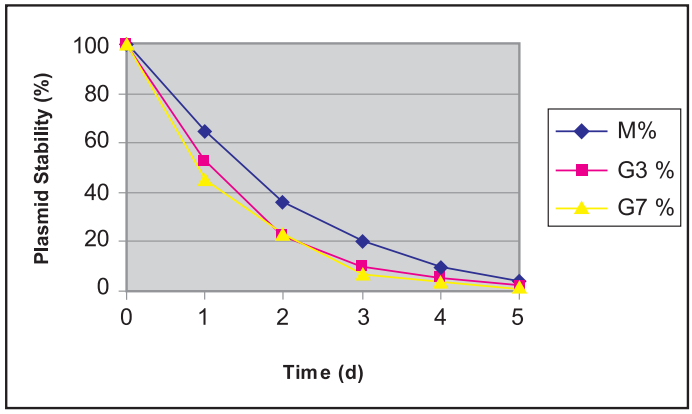

Figure 3. Stability of plasmid in *V. harveyi* strains M, G3, G7 under non-selective conditions.

Plasmid stability

Colonies grown on antibiotic-containing media exhibited uniform fluorescence appearance, but those grown on media without antibiotic showed mixture of fluorescent and non fluorescent colonies which might indicate plasmid loss. The stability of the GFP-plasmid construct was investigated during sequential propagation in the absence of antibiotic selection for five successive days (Fig. 3). Under non selective long term experiments without antibiotic pressure, pWG01 was highly unstable. Therefore transposon insertion may provide an alternative method to insert *gfp* gene directly into the genomic DNA of *V. harveyi* in order to yield stable recombinants.

Plasmid burden

Since differences in the growth rate occurred due to the metabolic load on the cells caused by the need to replicate the plasmid, and to express its gene; then the growth of the cells with and without GFP plasmid in liquid SWC medium, was assayed. The results showed that under laboratory conditions, the growth rate of *V. harveyi* with or without the GFP expressing plasmid were almost identical (Fig. 4), indicating that there was no significant burden to the cells carrying these plasmids.

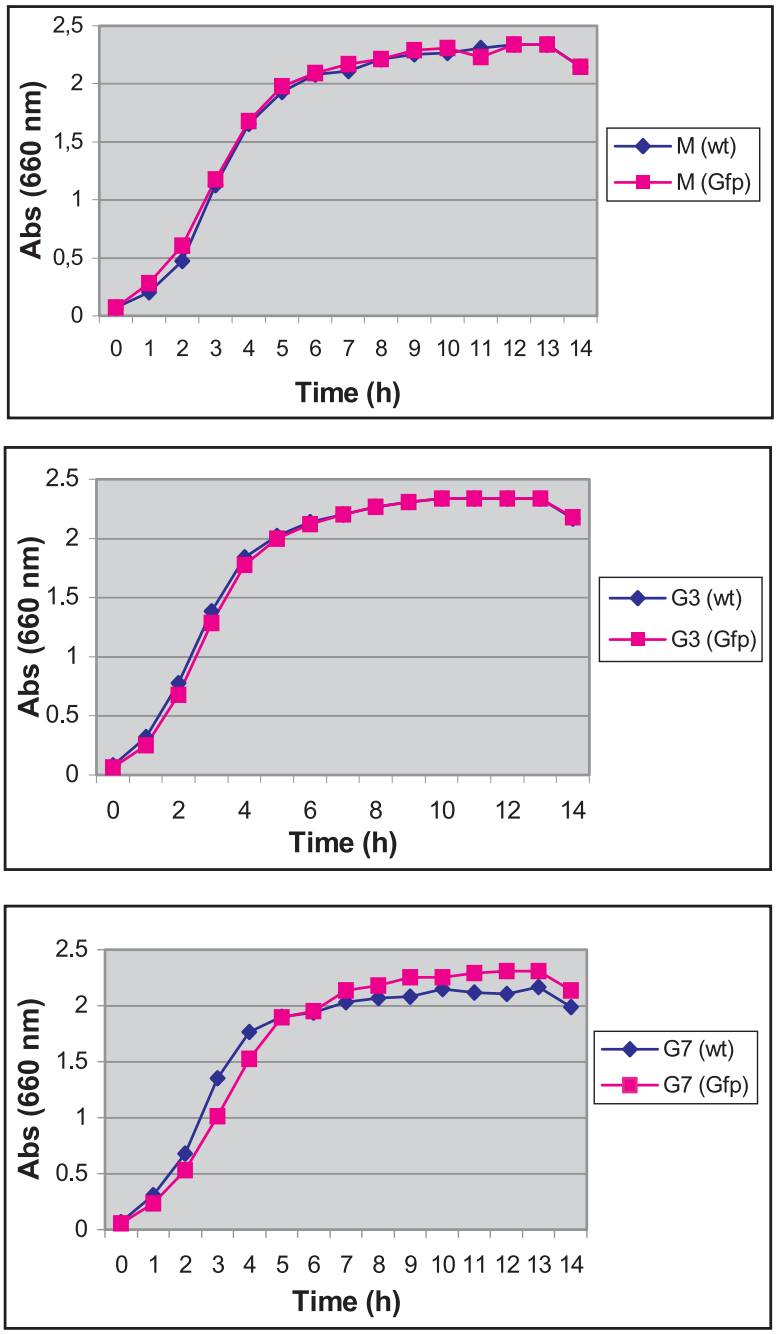

Figure 4. Growth of recombinant *V. harveyi* with or without GFP plasmid.

Adherence and pathogenicity assays

At day 5 after infection, cumulative mortalities in 20 experimentally infected shrimp larvae by *V. harveyi* pWG01 and wild type showed no significant differences (Table 2). This result indicated that the existence of GFP plasmid in *V. harveyi* did not significantly influence pathogenicity of *V. harveyi*.

Samples from dead shrimp larvae inoculated onto SWC plates with or without antibiotic showed that the dead shrimp larvae were infected by *V. harveyi*. Fluorescent *V. harveyi* (pWG01) was also directly observed in the carcasses of dead shrimp larvae (Fig. 2). Since pathogenic bacteria need to survive at the adhesion site and to escape from the host defence system, further studies to elucidate the mechanism of invasion of the adhered *V. harveyi* into the host tissues of shrimp larvae are in progress in our laboratory.

Table 2. Mortality (%) of shrimp larvae after immersed with fluorescent *V. harveyi* (pWG01), *V. harveyi* (wild type), and control.

Strains	Number of challenged larvae	Dead larvae	Mortality (%)
V. harveyi M (pWG01)	20	8.3	42
V. harveyi M (wild type)	20	8.3	42
V. harveyi G3 (pWG01)	20	8.7	43
V. harveyi G3 (wild type)	20	8.7	43
V. harveyi G7 (pWG01)	20	9.7	48
V. harveyi G7 (wild type)	20	10.0	50
Control (Broth only)	20	2.0	10

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REFERENCES

Bloemberg, G.V., O'Toole, G. A., Lugtenberg, B.J.J. and Kolter, R. 1997. Green fluorescent protein as a marker for *Pseudomonas* spp. Applied and Environmental Microbiology 63, 4543-4551.

Geoffroy, M.C., Guyard, C., Quatannens, B. Pavan, S. Lange, M. and Mercenier. A. 2000. Use of green fluorescent protein to tag lactic acid bacterium strains under development as live vaccine vectors. Applied and Environmental Microbiology 66, 383-391.

Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R. 1980. Broad-host range DNA cloning system for Gram negative bacteria: construction of gene bank of *Rhizobium meliloti*. Proceedings of the National Academy of Science USA 77, 7347-7451.

Hameed, A.S.S. 1993. A study of the aerobic heterotrophic bacterial flora of hatchery reared eggs, larvae and post larvae of *Penaeus indicus*. Aquaculture 117, 195-204.

Josenhans, C., Friedrich, S. and Suerbaum, S. Green fluorescent protein as a novel reporter system in *Helicobacter* sp. FEMS Microbiological Letters 161, 263-273.

Karunasagar, I., Pai, R., Malathi, G. R. and Karunasagar, I. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture 128, 203-209.

Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop II, R.M. and Peterson, K.M. 1994. pBBR1MCS2: A broad-host range cloning vector. Biotechniques 16, 800-802.

Lavilla-Pitogo, C.R., Baticados, M.C.L., Cruz-Lacierda, E.R. and de la Pena L.D.. 1990. Occurrence of luminous bacterial diseases of *Penaeus monodon* larvae in the Philippines. Aquaculture 91, 1-13.

Ling, S.H.M., Wang, X. H., Xie, L., Lim, T.M. and Leung, K. Y. Use of fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in *in vivo* and *in vitro* fish models. Microbiology 146, 7-19.

Manefield, M., Harris, L., Rice, S.A., De Nys, R. and Kjelleberg. S. 2000. Inhibition of luminescence and virulence in the black tiger prawn (*Penaeus monodon*) pathogen *Vibrio harveyi* by intercellular signal antagonist. Applied and Environmental Microbiology 66, 2079-2084.

Manning, E. 1997. Glow fish: An unusual glowing molecule from jelly fish is helping to illuminate cellular events. Biosciences 47, 135-138.

Ruangpan, L.. 1998. Luminous bacteria associated with shrimp mortality. *In* Flegel, T.W. (ed.). Advances in Shrimp Biotechnology, National Center for Genetic Engeneering and Biotechnology, Bangkok.

Salyers, A. and Whitt, A. 1994. Bacterial Pathogenesis: A Molecular Approach. ASP Press, Washington D.C. pp, 30-46.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Stretton, S., Techkarnjanaruk, S., McLennan, A.M. and Goodman. A. E. 1998. Use of green fluorescent protein to tag and investigate gene expression in marine bacteria. Applied and Environmental Microbiology 64, 2554-2559.

Sukenda and Wakabayashi, H. 2001. Adherence and infectivity of green fluorescent protein-labelled *Pseudomonas plecoglosicida* to ayu (*Plecoglosus altivelis*). Fish Pathology 36, 161-167.

Suwanto, A., Yuhana, M., Herawaty, E. and Angka, S.L. 1998. Genetic diversity of luminous vibrio isolated from shrimp larvae. In T.W. Flegel (ed.) Advances in Shrimp Biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok, pp. 217-224.

Teo, J.W.P., Suwanto, A. and Poh, C.L. 2000. Novel β-lactamase genes from two environmental isolates of *Vibrio harveyi*. Antimicrobial Agents and Chemotherapy 44, 1309-1314.

Tjahjadi, M. R., Angka, S. L. and Suwanto. A. 1994. Isolation and evaluation of marine bacteria for biocontrol of luminous bacterial disease in tiger shrimp larvae (*Penaeus monodon*, Fab.). Asia Pacific Journal of Molecular Biology and Biotechnology 2, 347-352.

Verschuere, L., Rombaut, G., Sorgeloos, P. Verstraete, W. 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Reviews 64, 655-671.

Widanarni and Suwanto, A. 2000. Genetic diversity of ampicillin resistant *Vibrio* isolated from various stages of shrimp larvae development. Biotropia 15, 36-47.