BIOTROPIA VOL. 13 NO. 1, 2006 : 1 - 10

TRANSPOSITION AND EXPRESSION OF *GFP* GENE IN THE GENOME OF *Vibrio harveyi* TO MONITOR ITS ADHERENCE IN SHRIMP LARVAE

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ABSTRACT

Expression of green fluorescent protein encoded by *gfp* gene in *Vibrio harveyi* was investigated to understand the ability of the gene as a molecular marker for adherence of this pathogenic *Vibrio* in shrimp larvae. *The gfp* gene was inserted into pl/ClSNot and pUTmini-Tn.') to generate a recombinant plasmid pWG02 and p\VG03, respectively; which was transferred into the three isolates of *V. han'eyi* employing diparental mating. Recombinant *E. coli* carrying p\VG02 and pWG03 resulted in green-fluorescent colonies and cells due to the production of GFP. However, all of mini-Tn.J, including mini-Tn.5-gfp were not successfully transferred to *V. harveyi.* Therefore, we used mini-Tn/fl (pLOFKm-gfp) for inserting of *gfp* gene into *V. han'eyi* genome. Although we could obtain relatively high (10^{5}) transconjugans employing Tn/rt, only one of *TnJO* derived isolate of *V. harveyi* G3 (G3-Tn/flgfp) *showed gfp* expression and was further employed for adherence assay. Fluorescent G3-Tn70gfp cells could be observed inside the digestive tract of shrimp larvae and could be distinguished from *Vibrio* that naturally exist in shrimp larvae.

Key words: *gfp* gene, *Vibrio han'eyi,* gene expression, shrimp larvae, molecular marker

INTRODUCTION

Vibrio harveyi was identified as a causative agent of mass mortalities of shrimp larvae and were frequently associated with luminous *Vibrio* (Lavilla- Pitogo *et al.* \ 990; Karunasagar *et al.* 1994; Ruangpan 1998, Suwanto *et al.* 1998). Luminescent vibriosis in shrimp larvae is characterized by lethargy, anorexia, muscle opacity, bacterial masses in the hemocoel, and luminosity of the larvae (Lavilla-Pitogo *et al.* 1990).

Electron microscopy observation has revealed that bacteria colonize the feeding apparatus, forming bacterial plaques in heavily infected larvae, therefore, it is highly probable that the mouth is the main entrance for colonization of inner tissues (Lavilla-Pitogo *et al.* 1990).

Pathogenicity assays based on Koch's Postulates (Madigan *et al.* 2003) were practically difficult to be conducted in shrimp larvae due to their relatively small size and lack of availability of *Vibrio-free* larvae (Widanarni and Suwanto 2000). Investigations into adherence and pathogenicity processes of this disease might

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greatly facilitate if a visible marker could be introduced into the bacterial cells.

Pathogenic *V. harveyi* have observed their attachment to crustacean larvae by epifluorescence microscopy using 5-(4,6-dichorotriazin-2-yl) aminofluorescein (5-DTAF, D-16) as a marker (Soto-Rodriguez *et al.* 2003). 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 (Geoffroy *et al.* 2000).

A broad host-range plasmid expressing *gfp* gene (pWGOl) was constructed and has been successful to tag K *harveyi* (Widanarni *et al.* 2005). However, under non selective long- term experiments without antibiotic pressure, pWGOl 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.

In this report, we describe the construction in Tn vector and expression of *V. harveyi* carrying *gfp* gene in the genomic DNAs to monitor its adherence in shrimp larvae.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study and their relevant characteristics are described in Table 1. *Escherichia 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) and 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 pWG02 which has the *lac* promoter was constructed and used to drive the expression of *gfp.* The promoter and *gfp* gene were isolated from pSKLOl using £coRI sites and ligated into pUClSNot linearized with £coRI. The recombinant plasmid vector (pWG02) (Figure 1) was digested with *Notl* and then promoter and *gfp* gene were ligated into pUTmini-TnJ linearized with *Noil* resulting

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant characteristic (s)	Source/Reference
V. harveyi		
MR5339	Wild type	Maros Lab. collection
G3	Wild type	Gondol Lab. collection
G7	Wild type	Gondol Lab. collection
G3-Tn*10*gfp	G3::mini-Tn*10*Km*gfp*, Km^R, gfp^+	This study
E. coli		
DH5α	F', *lacZ*? M15, *recA*1, *hsdR*17	Sambrook *et al.* (1989)
S17-1(λ*pir*)	Pro^-, Res^-, Mod^+, *recA* integrated plasmid RP4-Tc::Mu-Km::Tn*7*	Herrero *et al.* (1990)
SM10 (λ*pir*)	*thi thr leu tonA lacY supE (λpir) recA*::RP4-2-Tc::Mu Km	Stretton *et al.* (1998)
Plasmid		
pSKL01	Gm^R, P_{lac}, gfp^+	Sukenda and Wakabayashi (2001)
pUC18Not	Identical to pUC18 but with *Not*I-polylinker of pUC18-*Not*I as MCS, Ap^R	Herrero *et al.* (1990)
pUTmini-Tn*5* Sp/Sm	Tn*5* derivative of chromosomal integration vector	de Lorenzo *et al.* (1990)
pLOFKm*gfp*	pLOFKm with promoterless *gfp* cloned *upstream* of *kan*, Km^R	Stretton *et al.* (1998)
pWG02	*gfp* gene of pSKL01 cloned into pUC18Not, Ap^R, gfp^+	This study
pWG03	*gfp* gene of pWG02 cloned into pUTmini-Tn*5* Sp/Sm, Ap^R, gfp^+, Sp/Sm^R	This study

in recombinant plasmid (pWGOS) (Figure 2). The recombinant plasmid vector was transformed into *E. colt* DH5a using a standard heat shock transformation (Sambrook *et al.* 1989) and the colonies carrying pWG02 and pWG03 were examined for green fluorescence under UV-trans-illuminator at 260 nm (Biometra Ti 1, Gottingen).

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

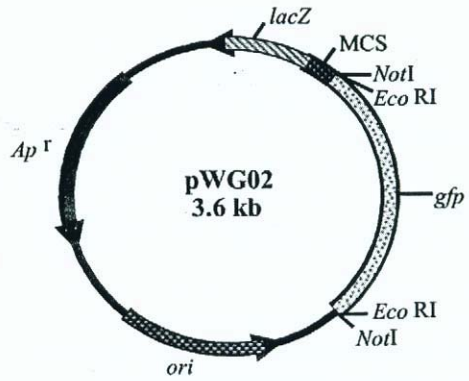


Figure 1. Construction of *gfp* carrier intermediate plasmid (pWG02)

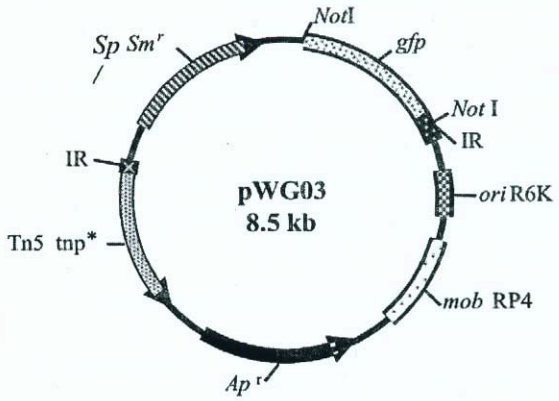


Figure 2. Construction of *gfp* carrier plasmid for transposition based of Tn5

Bacterial mating

To transfer recombinant plasmid pWG03 harboring mini-Tn5gfp into *V. harveyi* we used diparental mating (Suwanto and Kaplan 1992). *Escherichia coli* DH5a (pWGOS) donors were grown overnight in LB medium supplemented with spectinomycin and streptomycin (Sp/Sm) 50 ugmT[1] at 37°C; whereas *V. harveyi* recipients were grown in SWC medium at 28°C. Each 1.5 ml of the donor 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 ul of LB medium before being spotted onto a filter (1 cm diameter; pore size 0.45 Urn; Millipore) on LB medium agar. 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 on a vortex mixer.

The transconjugants were selected on Thiosulphate Citrate Bile Salt (TCBS, Oxoid) medium supplemented with Sp/Sm (50 jugm!"[1]). The selective medium TCBS was used to inhibit the growth of *E. coli*, while allowing *V. harveyi* transconjugants harboring TnJgfp (resistant to spectinomycin and streptomycin) to grow.

The same method was conducted to transfer recombinant plasmid pLOFKm-gfp (resistant to kanamycin) into *V. harveyi* and the transconjugants were selected on TCBS medium supplemented with kanamycin (100 (agml"[1]). Some of *V. harveyi* transconjugants were analyzed by Pulsed-Field Gel Electrophoresis (PFGE) with *Notl* restriction enzyme (Suwanto and Kaplan 1992; Widanarni and Suwanto 2000) to show the place of TnlOgfp inserted in the genomic of *V. harveyi.*

GFP stability and pathogenicity assay

Vibrio harveyi strains harboring *g/p* both in the plasmid pWGOl (Widanarni *et al*. 2005) and in the genomic's DNA were grown overnight in SWC broth supplemented with kanamycin. Sequential propagation under non selective conditions were performed by inoculating with 1:100 (v/v) to assess *gfp* existence by comparing duplicate colony counts on selective and non selective plates.

For pathogenicity assay, two groups with three duplicates of shrimp post-larvae (PL_4) were immersed for 30 min in 10^6 CFUml"[1] *of gfp* recombinants and wild type of *V. harveyi* (final concentration), respectively, and then placed in a 2 L shrimp rearing tank. A control group was immersed in sterile scawater. Daily survival rate of shrimp larvae for 5 days were recorded and compared with the control group.

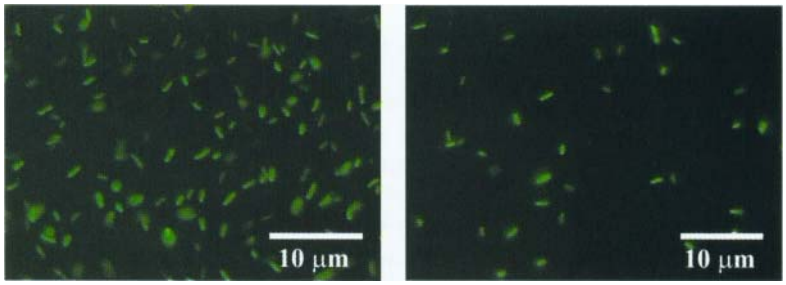
Adherence assay

Samples of shrimp larvae from control and treatment groups were directly observed under a fluorescence microscope. Samples from dead shrimp larvae were also inoculated onto SWC plates containing kanamycin (100 figrnl"[1]) to show that the dead shrimp larvae were infected by recombinant *V. harveyi.*

RESULTS AND **DISCUSSION**

GFP-containing plasmid construction

The GFP-plasmid vector was constructed for molecular marker in *V. harveyi.* Two GFP vectors i.e. pWG02 and pWGOS, were constructed with *lac* promoter to drive the expression *of gfp.* Recombinant *E. coli* carrying pWG02 or pWG03 (mini-TnJgfp) resulted in green-fluorescent colonies and cells due to the production of GFP (Figure 3). However, all of mini-Tn5, including mini-TnJ-gfp was not successfully transferred to *V. harveyi.* The same results were observed by Stretton e?



a b

Figure 3. Fluorescence micrograph of (a) *E. coli* DH5a (pWG02) and (b) *E. coli* DHSct (pWG03)

al. (1998). Therefore, we used mini-Tn70 (pLOFKm-gfp) that has been constructed by Stretton *et al.* (1998) for inserting *ofgfp* gene into *V. harveyi* genome.

Construction of *V. harveyi gfp*$^+$ employing mini-Tn/0 (pLOFKm-gfp)

We could obtain relatively high transconjugants employing *TnlO* (Table 2). Pulsed-Field Gel Electrophoresis (PFGE) analysis of some *V. harveyi* transconjugants demonstrated that Tn70gfp was randomly inserted in the gcnomic *V. harveyi* G3 (Figure 4). However, only one of *TnlO* derived isolate of *V. harveyi* G3 (G3-Tn70gfp) resulted in green-fluorescent colonies and cells due to the expression of GFP and that fluorescence levels qualitatively was almost the same with G3 (pWGOl) (Figure 5). This result occurred due to the fact that *gfp* in *pLOFKmq/p* was constructed promoterless, so its expression depended on promoter strength in the insertion site within *V. harveyi* genome.

Table 2. Frequency of Tn*10* based-*gfp* transfer to *V. harveyi* strains

Recipients	Number of transconjugants	Frequency (transconjugants/ recipients)	Number of green colonies
V. harveyi MR5339	378	2.3×10^{-7}	-
V. harveyi G3	924	5.5×10^{-7}	1
V. harveyi G7	154	9.2×10^{-8}	-

GFP stability and pathogenicity assay

Colonies of *V, harveyi* G3 (G3-Tn70gfp) that were grown on media with or without antibiotic exhibited uniform fluorescence appearance. This was not the case

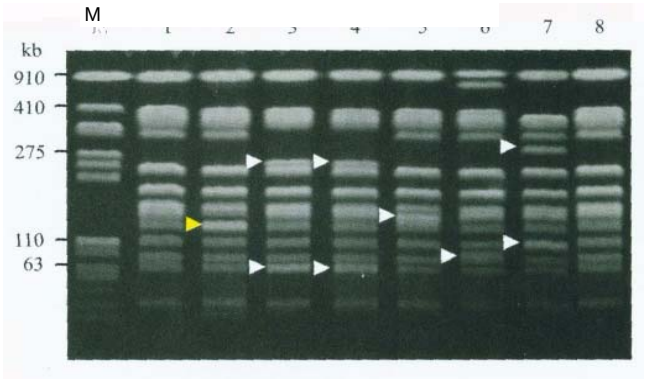


Figure 4. PFGE profiles of genomic DNA of *V. harveyi* G3 (Lane M: *Axel-digested* genotnic DNA of *R. sphaeroides* 2.4.1 as a molecular size marker (Suwanto and Kaplan 19 89). Lane 1 and 8: G3 wild type, Lane 2: G3 - Tn/flgfp (Km^R and showed *gfp* expression), Lane 3 -7: mutants 03 (Km^K)

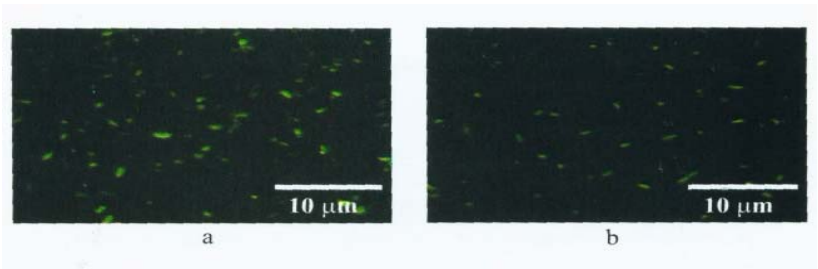


Figure 5. Fluorescence micrograph of (a) *V. harveyi* G3 (pWGOi) and (b) G3-Tn/Ogfp

for *V. harveyi* G3 (pWGOl). Their colonies grown on antibiotic-containing media exhibited uniform fluorescence appearance, whereas those grown on media without antibiotic showed mixture of fluorescent and non fluorescent colonies which might indicate plasmid loss. The stability of the GFP on *V. harveyi* G3 both in plasmid and in the genomic's DNA were investigated during sequential propagation in the absence of antibiotic selection for five successive days. Under non-selective long-term experiments without antibiotic pressure, pWGOl was highly unstable but Tn/0gfp was stably maintained in *V. harveyi* G3 strain (Figure 6). Insertion of Tn70gfp also did not show alteration in G3 pathogenicity to shrimp larvae (Figure 7), so that it was further employed for adherence assay.

Adherence assay

Sample from dead shrimp larvae showed that the dead larvae were infected by *V. harveyi.* *Vibrio harveyi* G3-Tn/0gfp could be isolated from dead shrimp larvae

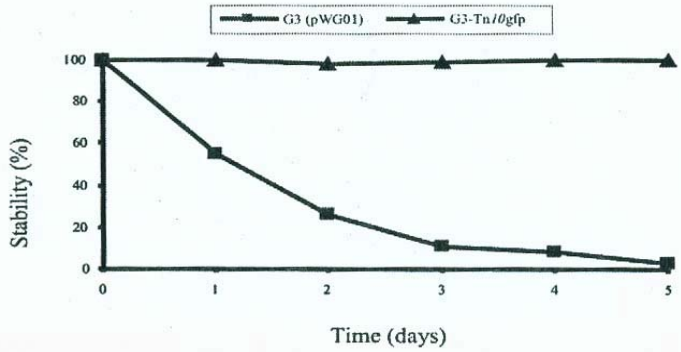


Figure 6. Stability of *gfp* in *V. harveyi* G3 (pWG01) and G3-Tn*10*gfp

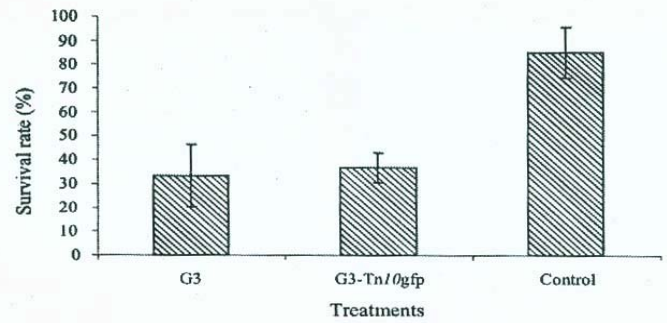


Figure 7. Survival rate of shrimp larvae on pathogen challenge assay of *V. harveyi* G3 and G3-Tn*10*gfp

placed on TCBS+Km media and fluorescent G3-Tn70gfp could also be observed directly in the carcasses of dead larvae. Fluorescent *G3-TnJOgfp* cells were observed in the oral region at 15-30 min after inoculation and could be observed inside the digestive tract at 2-3 h (Figure 8V The concentration of *V. harveyi* G3-*TnlOgfp* used in this study was 10 CFUml". Soto-Rodriquez *et al.* (2003) reported that 10^5 CFUml"^{1} *V. harveyi* labeled with *5-(4,6-dichlorotriazin-2-yl) aminofluorescein* (5-DTAF, D-16) could be observed in the oral region of *Litopenaeus vannamei* mysis at 0 and 2 h after ingestion. After 4 h inoculation, individual cells could already be seen inside the middle intestine, and at 18 or 24h, the fluorescent *V. harveyi* were observed throughout the intestinal tract. When presented at higher densities of bacterial cells (exclusively), fluorescent *V. harveyi* could be easily observed along all regions of zoea digestive tracts after 30 min (Soto-Rodriquez *et al.* 2003).

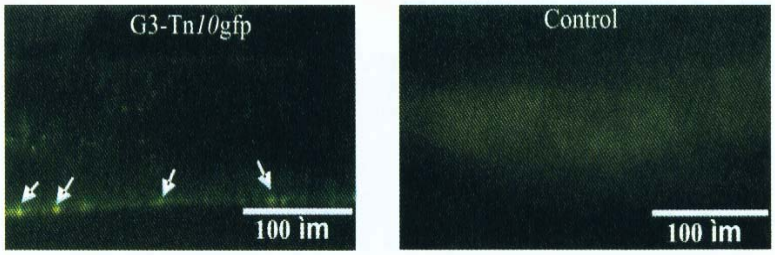


Figure 8. Observation of *V. harveyi* G3-Tn*10*gfp in the digestive tract of shrimp larvae

CONCLUSIONS

Recombinant *V. harveyi* harboring *gfp* both in plasmid pWGOl and in the genomic DNAs produced green-fluorescent cells. However, Tn7(?gfp was stably maintained in the genomic *V. harveyi* so that it could be used to monitor adherence and pathogenicity of *V. harveyi* in shrimp larvae.

ACKNOWLEDGMENTS

This work was supported by DIP-Funded Research of SEAMEO-B1OTROP (2000-2002) and International Foundation for Science grant (IPS, A/2207-2) to AS.

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