ADHERENCE AND PATHOGENICITY ASSAY OF VIBRIO HARVEYI IN TIGER SHRIMP (PENAEUS MONODON) LARVAE FOR SCREENING BIOCONTROL AGENT

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ABSTRACT

Rifampicin-resistant marker was employed as a reporter to detect the adherence and colonization of V. harveyi in shrimp larvae. Vibrio harveyi P1B and YA32.2 were isolated from dead shrimp larvae in Besuki, Northern Coast of East Java, while V. harveyi HB3, was isolated from pristine sea water in Pacitan, Southern Coast of East Java. Vibrio metchnikovii used as biocontrol agent was isolated from healthy shrimp larvae in Serang, West Java. Spontaneous mutation was conducted to generate V. harveyi P1B, YA32.2 and HB3 resistant to rifampicin. These mutants exhibited similar survival ability to their parental (wild type) strains. Significant larval mortality was observed in shrimp larvae inoculated with YA32.2 than that of larvae inoculated with P1B. Larvae inoculated with HB3 showed the lowest mortality. Bacterial cell count of Vibrio RP in dead larvae were 10^3-10^4 cells/larvae. Isolates of Vibrio metchnikovii Z and M as biocontrol candidates effectively reduced the growth and adherence ability of YA32.2 to shrimp larvae. Larval mortality in rearing water inoculated simultaneously with YA32.2 and V. metchnikovii was lower than the one inoculated with YA32.2 alone. Therefore, Vibrio metchnikovii Z or M could be developed as an effective probiotic or biocontrol agent for V. harveyi in shrimp hatcheries.

Key words: Biological control/Vibrio metchnikovii/shrimp larvae/Penaeus monodon/pathogenicity assay/Vibrio harveyi

INTRODUCTION

Shrimp farming is one of the most important activities in Indonesia and other Asian countries such as Thailand, Phillippines, and India (Ruangpan 1998). Up to 1997, Indonesia was the second largest world producer of tiger shrimp (Penaeus monodon) after Thailand with a production of more than 99 000 metric tons of tiger shrimp (Anonymous 1999). However, the exponential growth of shrimp culture in the last five years was not supported properly by a sufficient supply of shrimp larvae.
due to larval diseases and poor environmental quality of the hatcheries. Among shrimp diseases, vibriosis is one of fatal bacterial diseases. This disease is caused by *Vibrio* sp. that attacks the tiger shrimp at early larval or post-larval stage.

Mass mortality of shrimp larvae frequently associated with luminous *Vibrio* (Lavilla-Pitogo et al. 1998; Sunaryanto & Mariam 1986) was identified as *Vibrio harveyi* (Karunasagar et al. 1994; Lavilla-Pitogo et al. 1990). Although *Vibrio carchariae* was reported as a pathogen in a brown shark (*Carcharinus plumbeus*) that was found dead in captivity (Grimes et al. 1984), it has not been reported as a pathogen of shrimp larvae. It was also found associated with a chronic skin ulcer on a shark (Bertone et al. 1996).

So far, it has not been determined whether luminous *Vibrio* is a pathogen or saprophyte, even though it could frequently be isolated from dead larvae. Therefore, the pathogenicity status of this group of bacteria is uncertain. Pathogenicity assays based on Koch’s Postulate (Salyers & Whitt 1994) is practically difficult to be conducted in tiger shrimp larvae due to its relatively small size and since so far, no germ-free larvae are available (Hameed 1993). The source of luminous *Vibrio* colonized shrimp larvae might originate from the midgut contents of the spawners (Lavilla-Pitogo et al. 1992).

Bacterial pathogenicity is determined by several factors such as the ability to adhere to the host tissue to colonize and to secrete virulence factors (Salmond et al. 1995). The aim of this study was to determine the adherence and colonization of *Vibrio* isolates and their pathogenicity to shrimp larvae. Bacterial isolates were molecularly tagged to distinguish it from *Vibrio* naturally associated with the shrimp larva. Luminous *Vibrio* isolated from hatcheries and coastal water in East Java, Indonesia, showed sensitivity to rifampicin (Tjahyadi et al. 1994). Therefore, we used rifampicin resistant (Rif<sup>R</sup>) *Vibrio* generated by spontaneous mutation as a marker for *V. harveyi* isolates used in this study. *Vibrio harveyi* P1B and YA32.2 were isolated from dead larvae, while HB3 was isolated from pristine sea water (Suwanto et al. 1998; Teo et al. 2000). Our study also indicates that adherence assay in this study could be developed to screen for potential biocontrol bacteria or probiotics against pathogenic *Vibrio*.

**MATERIALS AND METHOD**

**Isolation and Identification of Vibrio carchariae**

The dead shrimp larvae were obtained in February 1997 from Besuki in the Northern Coast of East Java, Indonesia. The larvae were rinsed twice with sterile sea water and placed aseptically on the thiosulphate citrate bile salt (TCBS) agar. The plate was incubated for 24 h at room temperature (28 ± 2 °C). Isolated luminous colonies were restreaked on *Vibrio harveyi* agar (VHA) (Harris et al. 1996).
Bacterial characterization was conducted employing cellular fatty acid analyses by Microcheck, Inc., Microbial Analyses Laboratory in Northfield, USA. Bacteria were grown on trypticase soy broth agar (TSBA) at 28 °C and then extracted for cellular fatty acid analysis (Miller 1982). The fatty acid composition of each isolate was compared to a database of standard strain (V. carchariae ATCC 35084) profile, using overlap coefficient which allows their identification according to their similarity index.

Two other V. harveyi isolates used in this study were isolated and characterized from dead larvae (P1B) and from pristine sea water (HB3) (Sawanto et al. 1998).

Antibiotics Sensitivity

In order to tag *Vibrio* with the antibiotics resistant markers, it is necessary to determine the natural antibiotic sensitivity of the isolates. All isolates used in this research were tested for antibiotic sensitivity against kanamycin (50 μg/ml), ampicillin (50 μg/ml), tetracycline (10 μg/ml), gentamicin (20 μg/ml) and rifampicin (10 μg/ml) on Luria Bertani (LB) agar (10 g NaCl, 10 g tryptone, 0.5 g yeast extract, 15 g agar and 1 L distilled water).

Rifampicin-Resistant *Vibrio harveyi*

Spontaneous mutants of *V. harveyi* resistant to rifampicin (Rf<sup>R</sup>) were selected on LB agar supplemented with rifampicin (50 μg/ml) (Eisenstad et al. 1994). The survival of Rf<sup>R</sup> *V. harveyi* was evaluated and compared to the wild type strains in artificial larval-rearing water (3% NaCl and 0.3% yeast extract in 1L distilled water: SYE).

Growth Pattern and Survival Assay

*Vibrio harveyi* Rf<sup>R</sup> was inoculated into 100 ml SYE media and incubated at 28°C. Cell concentration was enumerated daily on TCBS and TCBS supplemented with 50 μg/ml Rf (TCBS-Rf). Enumeration began just after *V. harveyi* was inoculated into SYE media. The survival of *V. harveyi* on TCBS was compared with the survival on TCBS-Rf to detect the stability of the Rf<sup>R</sup> mutation.

Preparation of Shrimp Larvae

Two-day-old tiger shrimp postlarvae (PL2) and sea water for larval rearing were obtained from a hatchery in Labuhan, West Java. The larvae were maintained for acclimatization in a three-liter glass stock jar for at least two days in laboratory condition. Sea water salinity was maintained at 2.5% and at a temperature of 28-30°C. The larvae were fed on an artificial diet (Lancy PL, Inve aquaculture, Belgium). For adherence and pathogenicity assays, 50 larvae were kept in a three-liter glass jar containing 2.5 liters of autoclave-sterilized sea water.
Adherence Assay

Tested bacterial strains used in the adherence assays were cultured in 50% sea water complex (SWC) agar medium (pH 7.2) for 24 h at 28 °C. The culture was harvested by adding 5 ml sterile sea water onto the bacterial lawn, scraped, and collected into a tube, and then centrifuged at 5000 x g for two minutes. The bacterial cells were rinsed in sterile sea water and diluted to give a final concentration of 10^7 CFU/ml. The number of wild type Vibrio and Vibrio Rif^R in larval rearing water was enumerated daily on TCBS and TCBS-Rif, respectively. The number of larval mortality was counted daily. The Vibrio harveyi which colonized the dead larvae was enumerated daily on TCBS-Rif.

Challenge Test

The isolates used for the challenge test in this study were Vibrio metschnikovii isolates Z and M, which were isolated from healthy zoea (Z) and mysis (M), respectively (Widanarni 1999). Cell suspension of Vibrio metschnikovii Z and M at a concentration of 10^9 CFU/ml were inoculated into larval rearing jar 2 h before the shrimp larvae were introduced into the jar. After 6 h cocultivation of Z or M isolate with shrimp larvae, pathogenic Vibrio (YA32.2 Rif^R) were inoculated into the larval rearing jar. The treatments consisted of YA32.2 Rif^R which was challenged with V. metschnikovii Z, M, or a combination of Z and M. Shrimp larvae cocultivated with YA32.2 Rif^R alone was employed as a control. The number of larval mortality as well as concentration of wild type Vibrio and Vibrio Rif^R in the dead larvae and in rearing water were enumerated daily after cocultivation. The total number of Vibrio was enumerated on TCBS agar, while Vibrio Rif^R was counted on TCBS-Rif. Each experiment was conducted in duplicates. The number of dead larvae and bacterial cell concentration were presented in average values.

RESULTS

Isolation and Identification of Vibrio carlsmithiae

Two Vibrio isolates (YA32.2 and YA31.4) were isolated from dead larvae. Colonies of these isolates were luminous, circular and appeared green with dark green in the center of the colony when grown on TCBS agar. They also grew on VHA, a differential agar for Vibrio harveyi (Harris et al. 1996), and appeared as blue colonies due to decarboxylation of ornithin which shifted pH medium toward alkaline. When illuminated from below, yellow halos appeared, indicating cellulose fermentation around the colonies. Based on these specific characteristics, YA32.2 and YA31.4 were identified as V. harveyi.
Cellular fatty acid analyses of YA32.2 and YA31.4 grown on trypticase soybroth agar indicated that similarity index to \textit{V. carchariae} ATCC 35084 were 0.955 and 0.944, respectively. These similarity indices were much higher than those of \textit{V. parahaemolyticus} or \textit{V. alginolyticus} (Table 1). However Bergey’s Manual classifies \textit{V. carchariae} as a junior synonym (variant) to \textit{V. harveyi} (Baumann et al. 1994).

Four \textit{Vibrio harveyi} isolates used in this study, i.e.: P1B, HB3, YA32.2 and YA31.2, were resistant to ampicillin (50 µg/ml) and kanamycin (50 µg/ml) but sensitive to tetracycline (10 µg/ml), gentamicin (20 µg/ml) and rifampicin (10 µg/ml) (Table 2).

**Vibrio harveyi Mutant Rf³**

An amount of 50-100 µl of 10⁶ CFU/ml \textit{V. harveyi} was inoculated onto LB agar supplemented with 50 µg/ml rifampicin. Isolated colony which showed similar morphology and luminescence to the wild type was further employed for survival assay. The growth pattern and survival of three mutants P1B Rf³, YA32.2 Rf³ and HB3 Rf³ were similar to their parental wild type strains (data not shown).

<table>
<thead>
<tr>
<th>Vibrio sp</th>
<th>YA32.2</th>
<th>YA31.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{V. carchariae}</td>
<td>0.955</td>
<td>0.944</td>
</tr>
<tr>
<td>\textit{V. parahaemolyticus}</td>
<td>0.928</td>
<td>0.921</td>
</tr>
<tr>
<td>\textit{V. parahaemolyticus} sub group B</td>
<td>0.928</td>
<td>0.921</td>
</tr>
<tr>
<td>\textit{V. parahaemolyticus} sub group A</td>
<td>0.775</td>
<td>0.807</td>
</tr>
<tr>
<td>\textit{V. alginolyticus}</td>
<td>0.892</td>
<td>0.888</td>
</tr>
<tr>
<td>\textit{V. alginolyticus} sub group A</td>
<td>0.892</td>
<td>0.888</td>
</tr>
<tr>
<td>\textit{V. alginolyticus} sub group B</td>
<td>0.774</td>
<td>0.812</td>
</tr>
<tr>
<td>\textit{Leiognathia}</td>
<td>0.880</td>
<td>0.887</td>
</tr>
<tr>
<td>\textit{L. anguillarum}</td>
<td>0.880</td>
<td>0.887</td>
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<tr>
<td>\textit{Photobacterium}</td>
<td>0.533</td>
<td>0.660</td>
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<td>\textit{P. damselae}</td>
<td>0.533</td>
<td>0.660</td>
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<tr>
<td>\textit{P. leiognathi}</td>
<td>0.315</td>
<td>0.378</td>
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**Remarks:** Analyses were conducted by Microcheck, Inc. Microbial Analysis Laboratory Northfield, New Hampshire.
Table 2. Inhibition zone of antibiotics to the growth of four *Vibrio harveyi* isolates used

<table>
<thead>
<tr>
<th>Antibiotics Concentration (µg/ml)</th>
<th>P1B</th>
<th>HB3</th>
<th>YA32.2</th>
<th>YA31.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td></td>
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<tr>
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<td>0</td>
<td>0</td>
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<td>50</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>100</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Kanamycin</td>
<td></td>
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<td></td>
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<tr>
<td>25</td>
<td>0</td>
<td>0</td>
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<td>50</td>
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<td>100</td>
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<td>1</td>
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<tr>
<td>Tetracycline</td>
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<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>10</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
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<td>20</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Gentamicin</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>10</td>
<td>0</td>
<td>0</td>
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<td>20</td>
<td>2</td>
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<tr>
<td>25</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Rifampicin</td>
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<tr>
<td>10</td>
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<td>20</td>
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<tr>
<td>25</td>
<td>2</td>
<td>3</td>
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<td>3</td>
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</tbody>
</table>

Adherence Assay

About $10^6$ CFU/ml of *V. harveyi* Rif was inoculated into larval rearing water for adherence assay on shrimp larvae. Bacterial growth profile in larval rearing water showed similar patterns to that of the wild type (Fig. 1). The three strains showed bacterial growth patterns that generally increased in cell concentration at first to second or third day and decreased the following days. The highest concentration of *Vibrio* Rif in larval-rearing water was approximately $10^7$ CFU/ml.

Larval mortality in response to the three *Vibrio* Rif strains was different as presented in Figure 2. Isolate YA32.2 caused the highest larval mortality on the first day and the number of *Vibrio* Rif found in the dead larvae was $2.3 \times 10^5$ CFU/larvae. On the second day after inoculation, the number of dead larvae increased to 36 animals concomitant with the highest *Vibrio* Rif concentration at $1.2 \times 10^7$ CFU/larvae. All of the larvae were found dead on the third day with *Vibrio* Rif found in the larvae at concentration of $1.3 \times 10^5$ CFU/larvae.
Figure 1. Comparison of *Vibrio RF* and their respective parental wild type growth pattern in the larval rearing water.
Figure 2. Correlation between the cumulative number of death larvae and the number of Vibrion adhered in the shrimp larvae
Larval mortality in the jars inoculated with P1B occurred on the second day after inoculation. The number of dead larvae found on the second day was 19 animals. Larval mortality reached its highest number on the third day when the concentration of *Vibrio RI* in the larvae was $8.7 \times 10^3$ CFU/larva. The lowest larval mortality was found in the larvae inoculated with HB3. Larval mortality was found only after three days of coinoculation, and until the last day of observation, larval mortality occurred in only 13 animals carrying *Vibrio RI* at concentration of $1.75 \times 10^6$ CFU/larva (Fig. 3).

**Challenge Test**

Shrimp larvae inoculated with YA32.2 supplemented with *V. metschnikovii Z* or M, either alone or in combination, showed less mortality than the larvae treated only with YA32.2 alone as shown in Figure 4. The number of dead larvae when treated with YA32.2 in the presence of *V. metschnikovii M* was 11 larvae. Moreover, the number of dead larvae slightly increased if YA32.2 was inoculated in the presence of Z and M, although the number of *Vibrio RI* which adhered to the larvae was relatively similar i.e. $1.2 \times 10^7$ to $3.2 \times 10^7$ CFU/larva. All of the larvae treated with YA32.2 were found dead after three days of cocultivation when concentration of *Vibrio RI* in the larvae reached $10^6$ CFU/larva (Fig.4).

![Graph showing bacterial count](image-url)

**Figure 3.** Comparison between bacterial cell count of YA32.2 when challenged or when inoculated alone.
Figure 4. Correlation between the number of dead larvae and YA32.2 adherence in a challenge test experiment
DISCUSSION

Fatty acid profile analyses indicated that isolates YA32.2 and YA31.4 were V. 
charchariae. The results indicated that VHA media which was supposedly selective 
for V. harveyi (Harris et al. 1996) actually could also support the growth of V. 
charchariae, although DNA-DNA hybridization analysis suggested that V. 
charchariae is a junior synonym for V. harveyi (Baumann et al. 1994). In addition, 
rhotyping of V. harveyi and V. charchariae indicates very similar DNA banding 
patterns (Pedersen et al. 1998). Physiology and genetic similarity might explain the 
ability V. charchariae to grow on VHA media that was originally designed as a 
selective media for V. harveyi.

One of the physiological characteristics of V. charchariae is its capability to 
hydrolyze urea (UH\textsuperscript{+}). Other reports indicated that brown shark (from where 
the original name of V. charchariae was derived) excreted urea for osmoregulation. It 
was suggested, therefore, that the adherence of V. charchariae to shark was due to 
urea-urease linkage (Bertone et al. 1996). Meanwhile, urea hydrolyzing activity was 
reported as an indication of potential pathogenicity of V. paraaemolyticus (Kays-
ner et al. 1994). In V. paraaemolyticus, UH\textsuperscript{+} is related to hemolysin production, 
which is a virulence factor of Vibrio (Ozawa et al. 1996). The result of our study 
indicated that YA32.2, P1B and HB3 were UH\textsuperscript{+}, and two of them (i.e. P1B and 
YA32.2) were isolated from the dead shrimp larvae. However, the shrimps were 
reported to excrete ammonia, instead of urea (Baticados 1988). Therefore, 
the adherence of V. harveyi to shrimp might not be mediated by urea-urease linkage.

Three isolates used in this research demonstrated similar sensitivity to the test 
anitiotics. They were resistant to ampicillin and kanamycin but sensitive to tetracy-
cline, gentamicin and rifampicin. Vibrio charchariae isolated from shark skin ulcer 
was sensitive to tetracycline and resistant to piperacillin and carbenicillin (Bertone 
et al. 1996). Vibrio harveyi which was the causal agent for mass mortality of shrimp 
larvae in India, was sensitive to tetracycline but resistant to erythromycin. On 
the other hand, V. harveyi generally showed similar sensitivity to gentamicin (Austin 
et al. 1981) and rifampicin (Tjahyadi et al. 1994). This study indicated that V. harveyi 
P1B and HB3 and V. charchariae YA32.2 were sensitive to tetracycline, gentamicin 
and rifampicin. This result showed that V. harveyi isolated from anywhere has 
similarity in sensitivity to tetracycline, gentamicin and rifampicin.

Bacterial cell count of V. harveyi R\textsuperscript{f} in larval-rearing water showed a different 
number from that of Vibrio sp which occurred naturally in larval rearing water. The 
difference indicated the number of Vibrio sp originally colonized shrimp larvae. 
These Vibrio sp yielded green colonies which could not be distinguished from green 
colony of V. harveyi R\textsuperscript{f} in rearing water. Therefore, it is necessary to introduce 
molecular marker to distinguish the green colonies of Vibrio R\textsuperscript{f} from the green 
colonies of wild type Vibrio.
P1B, HB3 and YA32.2 were sensitive to Rf. Therefore, in this experiment, we used Rf as molecular marker to distinguish these three strains from the other V. harveyi isolates which occurred naturally in the shrimp larvae or larval rearing water. *Vibrio harveyi* Rf mutants showed similar growth patterns to that of the wild type strain. This result indicated that the survival of *V. harveyi* Rf was similar to the wild type, and Rf character was stable even when it was kept in larval-rearing water.

Although the cell densities of each *Vibrio* Rf in larval-rearing water were similar at approximately 10^7 CFU/ml (Fig. 1), larval mortality in response to the three Rf strains was different. The fastest and the highest larval mortality was due to YA32.2 inoculation which could be detected as early as one day after coinoculation time. Larval mortality due to inoculation with P1B was detected after two days of coinoculation although the number of the dead larvae which resulted from those treatments was relatively similar. Probably, the two strains were potential pathogens because they were isolated from dead shrimp larvae. Similarly observed mortality was found when shrimp larvae were inoculated with similar density of *V. campbelli*-like bacterium (Hameed 1995). Our study showed that HB3 was not pathogenic. Larval mortality in HB3 inoculation jar maybe due to the high density of *Vibrio*. Non-pathogenic or very weak pathogenicity of HB3 might be the reflection of its habitat where HB3 was originally obtained from pristine sea water in Southern Coast of Java Island.

Isolate YA32.2 was then selected for challenge test due to its potential pathogenicity. *Vibrio metschnikovii* as a challenge strain is a *Vibrio* species which usually adheres and colonizes shrimp larvae until the stages of post-larval stage. We assumed the strains of *V. metschnikovii* are not pathogen because they do not produce virulence factors (Widanarni 1999). Austin et al. (1995) used *V. alginolyticus* as a probiotic strain for *A. salmonicida*, *V. anguillarum* and *V. ordalii*. Our results indicated that *V. metschnikovii* Z or M could significantly reduce the growth and adherence of YA32.2 in shrimp larvae and significantly reduce larval mortality. This result demonstrated potential application of the adherence assay to screen for potential probiotics or biocontrol bacteria in shrimp hatcheries.

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