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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 metschnikovii* 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* Rf^R in dead larvae were 10^3-10^4 cells/larvae. Isolates of *Vibrio metschnikovii* 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. metschnikovii* was lower than the one inoculated with YA32.2 alone. Therefore, *Vibrio metschnikovii* Z or M could be developed as an effective probiotic or biocontrol agent for *V. harveyi* in shrimp hatcheries.

Key words : Biological control/*Vibrio metschnikovii*/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

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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 larvae. 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 (Rf^R) *Vibrio* generated by spontaneus 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) (Suwanto *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 kanamicin (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^R) were selected on LB agar supplemented with rifampicin (50 μg/ml) (Eisenstad *et al.* 1994). The survival of Rf^R *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^R 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-Rif). 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-Rif to detect the stability of the Rf^R 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 (*Lanzy 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 10^6 CFU/ml. The number of wild type *Vibrio* and *Vibrio* Rf^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 10^8 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 Rf^R) were inoculated into the larval rearing jar. The treatments consisted of YA32.2 Rf^R which was challenged with *V. metschnikovii* Z, M, or a combination of Z and M. Shrimp larvae cocultivated with YA32.2 Rf^R alone was employed as a control. The number of larval mortality as well as concentration of wild type *Vibrio* and *Vibrio* Rf^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* Rf^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 carchariae*

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 cellobiose 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 *V. carchariae* ATCC 35084 were 0.955 and 0.944, respectively. These similarity indices were much higher than those of *V. parahaemolyticus* or *V. alginolyticus* (Table 1). However Bergey's Manual classifies *V. carchariae* as a junior synonym (variant) to *V. harveyi* (Baumann *et al.* 1994).

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

Vibrio harveyi Mutant Rf^R

An amount of 50-100 μl of 10^8 CFU/ml *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 Rf^R, YA32.2 Rf^R and HB3 Rf^R were similar to their parental wild type strains (data not shown).

Table 1. Similarity index isolates YA32.2 and YA31.4 with *Vibrio* sp based on cellular fatty acid profiles

Vibrio sp	YA32.2	YA31.4
Vibrio	0.955	0.944
V. carchariae	0.955	0.944
V. parahaemolyticus	0.928	0.921
V.p. sub group B	0.928	0.921
V.p sub group A	0.775	0.807
V.alginoliticus	0.892	0.888
V.a. sub group A	0.892	0.888
V.a. sub group B	0.774	0.812
Listonella	0.880	0.887
L. anguillarum)	0.880	0.887
Photobacterium	0.533	0.660
P. damsela	0.533	0.660
P. leiognathi	0.315	0.378

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

Antibiotics Concentration (• g/ml)	Inhibiton zone (mm)			
	P1B	HB3	YA32.2	YA31.4
Ampicillin				
25	0	0	0	0
50	0	0	0	0
100	1	0	1	2
Kanamicin				
25	0	0	0	0
50	0	0	0	0
100	1	1	1	0
Tetracycline				
5	0	0	0	0
10	2	3	2	2
20	2	3	3	3
Gentamicin				
10	0	0	0	0
20	2	2	1	2
25	2	3	2	2
Rifampicin				
10	1	2	1	1
20	2	2	2	2
25	2	3	2	3

Adherence Assay

About 10^6 CFU/ml of *V. harveyi* Rf^R 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* Rf^R in larval-rearing water was approximately 10^7 CFU/ml.

Larval mortality in response to the three *Vibrio* Rf^R 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* Rf^R found in the dead larvae was 2.3×10^3 CFU/larvae. On the second day after inoculation, the number of dead larvae increased to 36 animals concomitant with the highest *Vibrio* Rf^R concentration at 1.2×10^4 CFU/ larvae. All of the larvae were found dead on the third day with *Vibrio* Rf^R found in the larvae at concentration of 1.3×10^4 CFU/larvae .

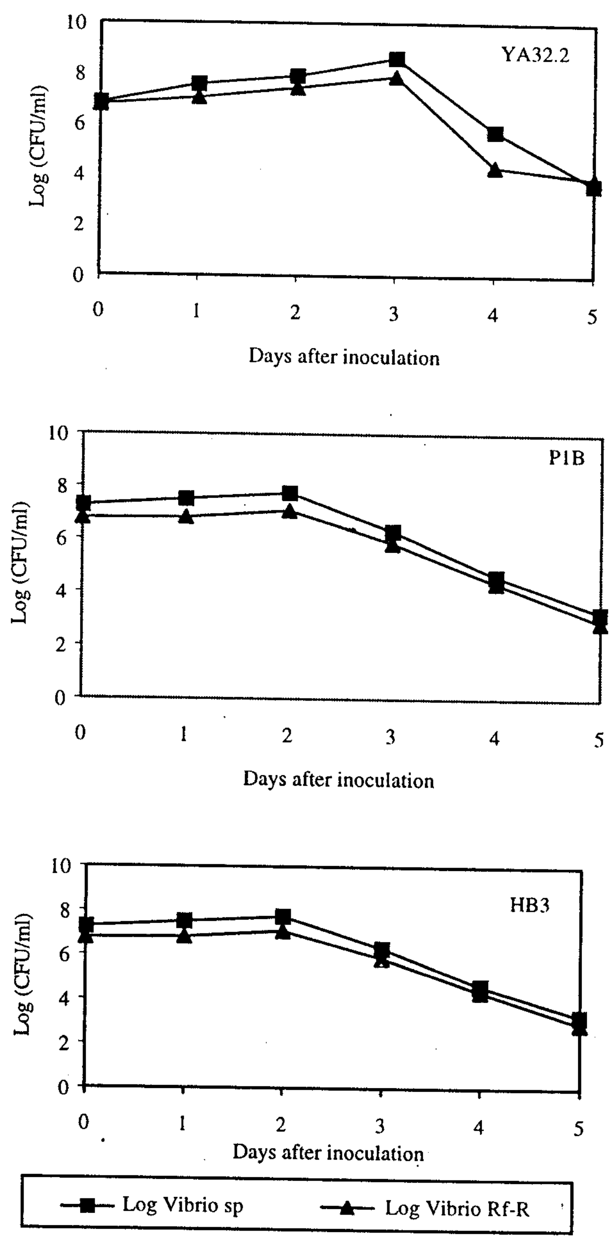

Figure 1. Comparison of *Vibrio* RF^R and their respective parental wild type growth pattern in the larval rearing water

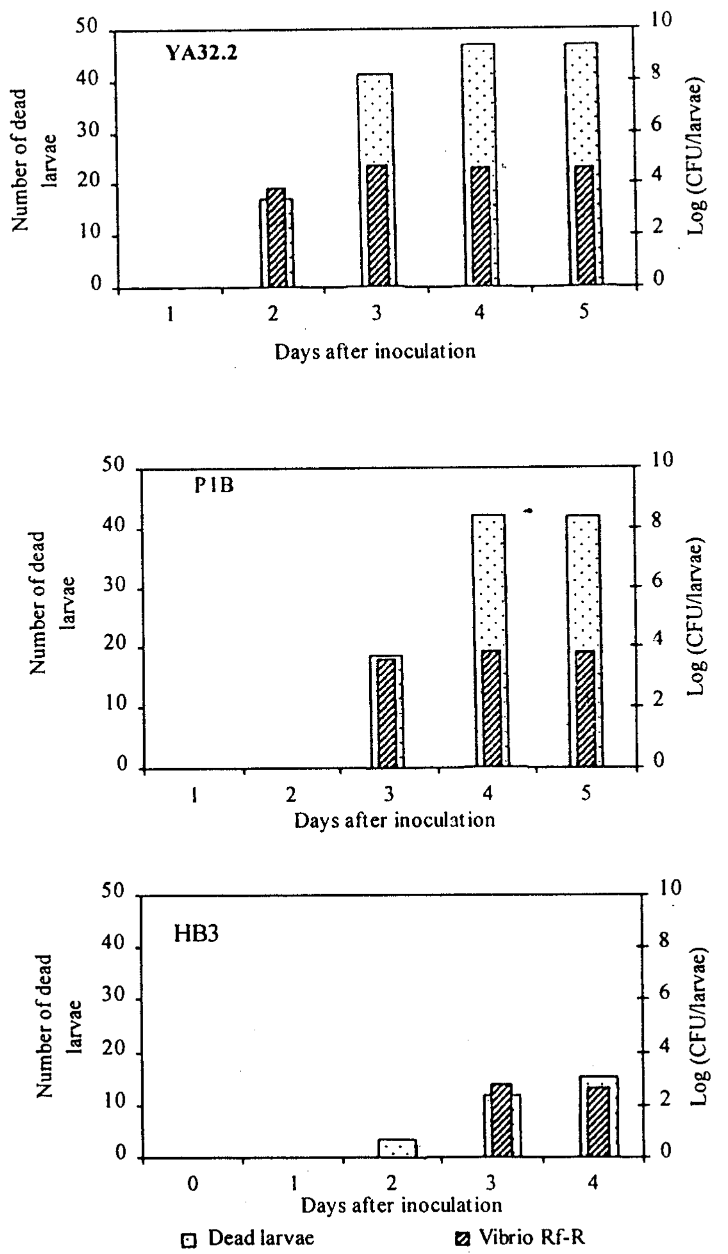

Figure 2. Correlation between the cumulative number of death larvae and the number of *Vibrio* adhered in the shrimp larvae

Larval mortality in the jars inoculated with P1B occured 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* Rf^R in the larvae was 8.7×10^3 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* Rf^R at concentration of 1.75×10^2 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* Rf^R which adhered to the larvae was relatively similar i.e. 1.2×10^2 to 3.2×10^2 CFU/larva. All of the larvae treated with YA32.2 were found dead after three days of cocultivation when concentration of *Vibrio* Rf^R in the larvae reached 10^4 CFU/larva (Fig.4).

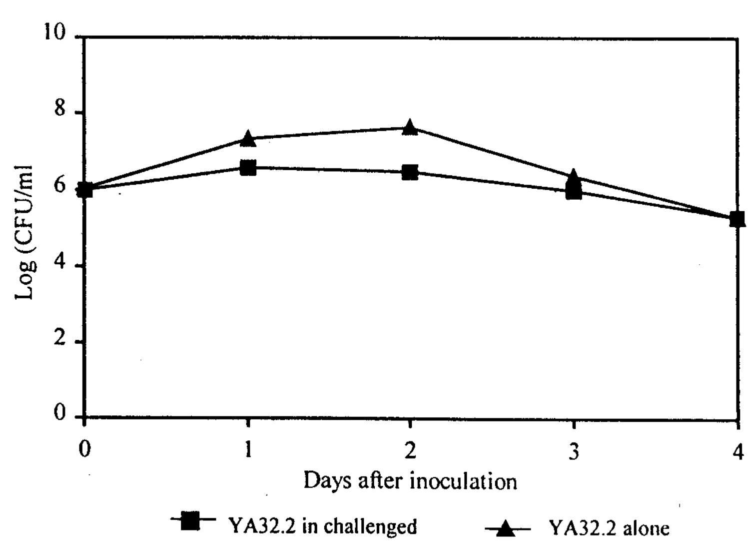

Figure 3. Comparison between bacterial cell count of YA32.2 when challenged or when inoculated alone

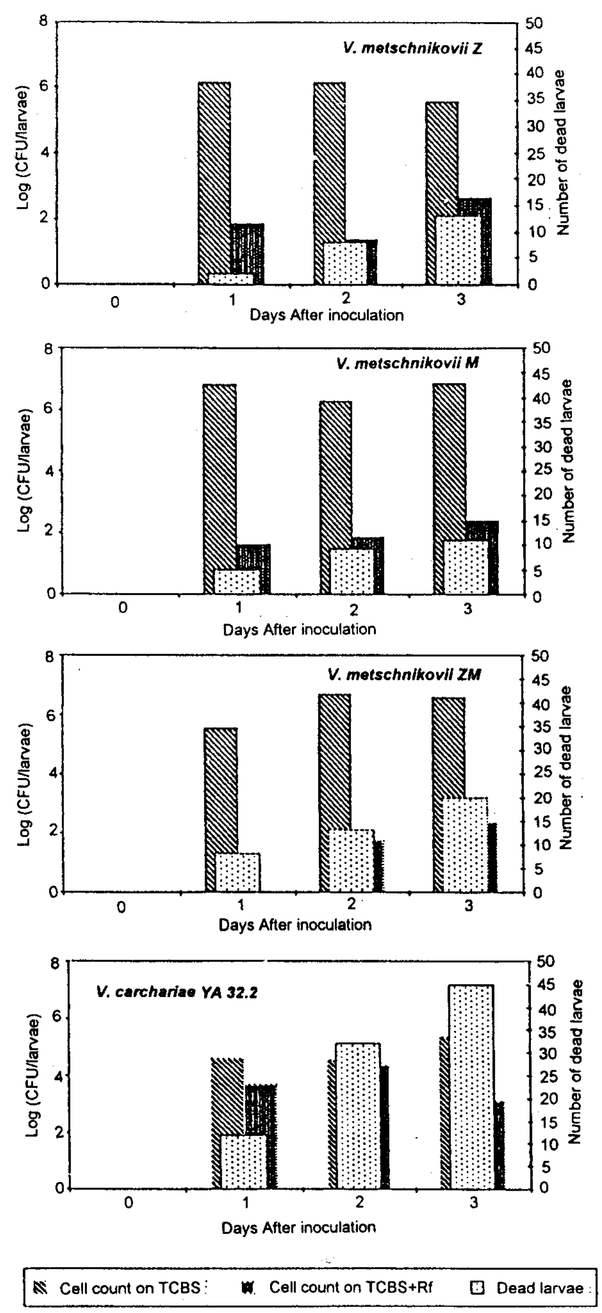

Figure 4. Correlation between the number of dead larvae and YA32.2 adherence in a challenge test experiment

DISCUSSION

Fatty acid profile analyses indicated that isolates YA32.2 and YA31.4 were *V. carchariae*. 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. carchariae,* although DNA-DNA hybridization analysis suggested that *V. carchariae* is a junior synonym for *V. harveyi* (Baumann *et al.* 1994). In addition, ribotyping of *V. harveyi* and *V. carchariae* indicates very similar DNA banding patterns (Pedersen *et al.* 1998). Physiology and genetic similarity might explain the ability *V. carchariae* to grow on VHA media that was originally designed as a selective media for *V. harveyi.*

One of the physiological characteristics of *V. carchariae* is its capability to hydrolyze urea (UH^+). Other reports indicated that brown shark (from where the original name of *V. carchariae* was derived) excreted urea for osmoregulation. It was suggested, therefore, that the adherence of *V. carchariae* 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. parahaemolyticus* (Kaysner et al. 1994). In *V. parahaemolitycus*, UH^+ is related to hemolysin production, which is a virulence factor of *Vibrio* (Osawa *et al.* 1996). The result of our study indicated that YA32.2, P1B and HB3 were UH^+, 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 antibiotics. They were resistant to ampicillin and kanamicin but sensitive to tetracycline, gentamicin and rifampicin. *Vibrio carchariae* 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. carchariae* 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* Rf^R 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* Rf^R in rearing water. Therefore, it is necessary to introduce molecular marker to distinguish the green colonies of *Vibrio* Rf^R from the green colonies of wild type *Vibrio*.

P1B, HB3 and YA32.2 were sensitive to Rf. Therefore, in this experiment, we used Rf^R 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^R mutants showed similar growth patterns to that of the wild type strain. This result indicated that the survival of *V. harveyi* Rf^R was similar to the wild type, and Rf^R character was stable even when it was kept in larval-rearing water.

Although the cell densities of each *Vibrio* Rf^R in larval-rearing water were similar at approximately 10^7 CFU/ml (Fig. 1), larval mortality in response to the three Rf^R 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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