Vol. 124: 223–232, 2017
https://doi.org/10.3354/dao03120

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published May 11

Isolation of *Bdellovibrio* and like organisms and potential to reduce acute hepatopancreatic necrosis disease caused by *Vibrio parahaemolyticus*

Jetnapang Kongrueng[1], Pimonsri Mitraparp-arthorn[1], Khotchawan Bangpanwimon[1], William Robins[2], V. Vuddhakul[1,*], John Mekalanos[2]

[1]Food Safety and Health Research Unit, Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Thailand

[2]Dept. of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115, USA

ABSTRACT: Acute hepatopancreatic necrosis disease, a severe disease of shrimp, is caused by *Vibrio parahaemolyticus* (AHPND Vp), a halophilic bacterium harboring a plasmid that contains toxin genes homologous to *Photorhabdus* insect-related toxins. We obtained 9 isolates of *Bdellovibrio* and like organisms (BALOs) from water and sediment samples in Thailand. Using 16S rRNA sequencing, all of the organisms were identified as *Bacteriovorax* spp. and were able to attack all tested AHPND Vp isolates. In addition, their various susceptible hosts, including Gram-positive and Gram-negative bacteria, were observed. The optimal ratio for interaction between the *Bacteriovorax* isolate BV-A and AHPND Vp was determined to be 1:10. The suitable conditions applied for co-culture between BV-A and AHPND Vp were 30°C, 2% NaCl, and pH 7.6. The capability of BV-A to reduce numbers of AHPND Vp *in vitro* was observed in co-culture after incubation for 2 d and continued until the end of the incubation period. *In vivo*, BV-A was able to reduce mortality of shrimp post-larvae infected with AHPND Vp. In addition, BV-A significantly decreased the formation of biofilm by AHPND Vp. These findings provide evidence for using *Bacteriovorax* as a biocontrol of AHPND Vp in shrimp aquaculture.

KEY WORDS: *Bdellovibrio* and like organisms · BALOs · *Bacteriovorax* · *Vibrio parahaemolyticus* · Acute hepatopancreatic necrosis disease · AHPND · Shrimp

INTRODUCTION

Shrimp aquaculture is an enterprise that has generated substantial export income for many countries. Previously, Thailand was the world's leading producer of cultured shrimp, followed by China, India, Ecuador, Vietnam, and Indonesia. However, in 2009, a new shrimp disease, named acute hepatopancreatic necrosis disease (AHPND), emerged in China and subsequently spread to Vietnam, Malaysia, Thailand, and Mexico (Tran et al. 2013, Joshi et al. 2014, Nunan et al. 2014). The causative agent of AHPND is *Vibrio parahaemolyticus*, a halophilic Gram-negative bacterium found in marine and estuarine environments (Tran et al. 2013). AHPND *V. parahaemolyticus* (AHPND Vp) harbors a unique large plasmid containing genes encoded for toxin homologs to the *Photorhabdus* insect-related toxins PirA and PirB (Yang et al. 2014). The bacterium affects post-larvae approximately 30 to 35 d after stocking the shrimp pond, causing massive rounding and sloughing of hepatopancreatic tubule epithelial cells in the early to mid stages of the disease (Hong et al. 2016). PirA is only present at the later stage, whereas PirB is detected in the hepatopancreas at the early stage of infection but is sufficient to cause cellular damage (Lai et al. 2015). This bacterium has been disastrous for the shrimp industry and has reduced shrimp production worldwide by up to 20%

*Corresponding author: varaporn.v@psu.ac.th

Publisher: Inter-Research · www.int-res.com

(Hong et al. 2016). Strenuous efforts to solve this problem have included cessation of shrimp cultivation and drying out of shrimp ponds for a period of time. However, the disease reoccurs after cultivation recommences. Therefore, pond management including biocontrol should be incorporated.

Bdellovibrio and like organisms (BALOs) are a group of small Gram-negative predatory bacteria that are ubiquitous in aquatic and terrestrial environments (Williams & Pineiro 2006). BALOs can invade the periplasm and multiply inside many prey bacteria, lysing them and further attacking other bacteria. This makes BALOs potentially powerful inhibitors of environmentally and clinically undesirable bacteria (Fratamico & Cooke 1996, Sockett & Lambert 2004). BALOs have been investigated as alternative organisms for the prevention and control of bacterial disease in aquaculture. *Bdellovibrio* F16, isolated from sturgeon gut, displayed bacteriolytic activity against the fish pathogen *Aeromonas hydrophila* (Cao et al. 2012). Fish challenged by immersion in water containing *A. hydrophila* and *Bdellovibrio* C-1 suffered lower mortality than fish in water containing *A. hydrophila* alone, suggesting the possibility of using *Bdellovibrio* to control this bacterium in fish (Chu & Zhu 2010). Two marine BALOs isolated from the sediment of a bay in China reduced total vibrios and *V. parahaemolyticus* in water and oyster intestine (Li et al. 2011). BALO strain BDHSH06 reduced the numbers of total bacteria and vibrios in pond water and shrimp intestine (Li et al. 2014). To solve the problem of drug resistance, which causes complications in bacterial infection, 2 genera of BALOs (*Bdellovibrio* and *Micavibrio*) have been demonstrated to be capable of attacking many human pathogenic bacteria such as *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Shigella*, and *Yersinia* (Dashiff et al. 2011). Although BALOs attack bacteria nonspecifically, they preferentially select prey to different degrees (Williams & Pineiro 2006). Therefore, it is of interest to investigate the potential of BALOs to eliminate AHPND Vp. The aims of this work were to isolate BALOs from various samples collected from AHPND shrimp ponds and to evaluate their potential to control the numbers of AHPND Vp *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

AHPND Vp designated as PSU 5429, PSU 5499, PSU 5562, and PSU 5579 were isolated from AHPND-afflicted shrimp collected from various farms in southern Thailand (Kongrueng et al. 2015) and were used as mixed prey bacteria to isolate BALOs. AHPND Vp PSU 5429 was used as a host prey for other assays throughout this study. Each bacterium was grown in Luria-Bertani (LB) broth supplemented with 1% NaCl at 30°C with shaking at 150 rpm for 3 h and adjusted to 10^8 CFU ml^{-1} using a densitometer (Densimat, bioMérieux). The bacterial cells were then spun down, and LB broth was replaced with diluted nutrient broth (DNB). Each 1 ml of bacterial strain was mixed and used as a prey cocktail to isolate BALOs.

Isolation and purification of BALOs

Water and sediment field samples were collected off an island and from AHPND Vp-infected shrimp farms located in central and southern Thailand. Isolation of BALOs was performed using the double-layered plaque assay (Medina et al. 2008). Briefly, in the pre-enrichment step, a test sample of either 10 g or 10 ml of sediment or water was mixed with 100 ml of DNB (Starr 1975) and 1 ml of prey cocktail (10^8 CFU ml^{-1}). The mixture was incubated with shaking (200 rpm) at 30°C for 7 d; it was then centrifuged and the supernatant was passed through a 0.45 µm membrane filter (Millipore). The filtrate was concentrated by centrifugation at 22 000 × *g* for 1 h, and the pellet was re-suspended in DNB and mixed with 300 µl of prey cocktail (10^8 CFU ml^{-1}); it was then overlaid on diluted nutrient agar (DNAg). The plate was incubated at 30°C, and the development of plaque formation within 3 to 7 d indicated the presence of BALOs. Individual plaques were purified by 3 passages, and the BALOs were cultivated in DNB with AHPND Vp (PSU 5429) to enhance the titer and kept at –80°C with 10% glycerol.

For any assay, each BALO was recovered from the stock and its titer was enhanced as described above. To enumerate the BALOs, a 10-fold dilution of BALO suspension was performed, and 100 µl of each dilution were mixed with 4 ml of soft DNAg (0.6% agar) containing 300 µl of AHPND Vp PSU 5429 at a concentration of 10^8 CFU ml^{-1}. The mixture was then overlaid on a DNAg plate and incubated at 30°C. The numbers of plaques were counted after at least 3 d of incubation.

Molecular identification

To identify the genus of the BALOs, genomic DNA of BALOs was extracted by boiling. The 16S rRNA

gene was amplified by PCR using primers specific to the *Bdellovibrio* 16S rRNA gene (63F primer: 5′-GAG GCC TAA CAC ATG CAA GTC-3′; 842R primer: 5′-CGW CAC TGA AGG GGT CAA-3′) (Jurkevitch et al. 2000) and the *Bacteriovorax* 16S rRNA gene (Bac676F primer: 5′-ATT TCG CAT GTA GGG GTA-3′; Bac1442R primer: 5′-GCC ACG GTT CAG GTA AG-3′) (Davidov et al. 2006). The PCR product was electrophoresed on 1% agarose gel and was detected using a UV transilluminator. For sequencing, the PCR product was purified using a Qiagen kit and sequenced.

Phylogenetic tree analysis

The 16S rRNA sequences of BALOs obtained in this study were analyzed against the database at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) program. The sequences of BALO isolates were aligned by ClustalW with reference strains. The aligned sequences were analyzed using MEGA 6.0 software (Tamura et al. 2013). The phylogenetic tree was constructed by neighbor joining using the TN93 model (Tamura & Nei 1993). Statistical significance levels of interior nodes were determined by bootstrap analysis with 1000 replications.

Susceptibility of Gram-positive and Gram-negative bacteria to BALOs

The ability of BALOs to attack both Gram-positive and Gram-negative bacteria was investigated with *Staphylococcus aureus*, *Escherichia coli*, *Vibrio vulnificus, V. cholerae, V. alginolyticus,* AHPND Vp, and clinical and environmental *V. parahaemolyticus* isolates. A bacterial lawn was produced by shaking a loop full of each bacterium in LB broth for 3 h before mixing with melted DNAg and spreading on DNAg. To increase the titer of BALOs, each pure culture of BALO strain in DNB was mixed with AHPND Vp PSU 5429 and incubated with shaking at 30°C for 3 d. The mixture was then filtered to remove the host bacterium, and 10 µl of the filtrate were dropped on the plate containing the bacterial lawn. A clear zone was observed after incubation at 30°C for 3 to 7 d. All experiments were performed in triplicate independently, and at least 1 positive out of the 3 experiments was defined as susceptible, whereas 3 negative experiments indicated non-susceptibility.

Optimal conditions of interaction between a BALO strain and AHPND Vp

To assess factors that affect the ability of a BALO to inhibit AHPND Vp, we evaluated the multiplicity of infection of a selected BALO isolate and an AHPND Vp isolate including temperature, salinity, and pH. Each 1 ml of a BALO designated as BV-A at concentrations of 10^3 to 10^6 plaque-forming units (PFU) ml^{-1} was incubated with 1 ml AHPND Vp PSU 5429 suspension (10^7 CFU ml^{-1}) in 100 ml DNB at a ratio between 1:10 and 1:10 000. This co-culture was incubated with shaking at 30°C for 7 d, and the numbers of AHPND Vp were enumerated daily by the plate count technique. To investigate the effect of temperature, the co-culture was incubated with shaking at 25, 30, and 37°C, and for salinity and pH evaluations, the co-culture was inoculated in DNB supplemented with 1 to 6% NaCl at pH between 7 and 9. For controls, general shrimp pond conditions were applied, AHPND Vp was inoculated in DNB supplemented with 2% NaCl, pH 7.6, and incubated with shaking at 30°C. The experiment was performed in triplicate. The reduction of *V. parahaemolyticus* was calculated based on the percent difference between the initial numbers of the bacterium in the control before and after treatment.

Potential of a BALO strain to suppress AHPND Vp *in vitro*

Co-culture of a BALO strain and an AHPND Vp isolate was performed in the optimal conditions described above for 7 d. A control for each organism was included. The numbers of BALO and AHPND Vp were enumerated daily using double-layer agar and spread plate techniques, respectively.

Efficacy of a BALO strain to inhibit AHPND Vp in shrimp larvae

To assess the ability of a BALO strain to reduce mortality in shrimp larvae infected with AHPND Vp, whiteleg shrimp *Litopenaeus vannamei* post-larvae (PL24) were obtained from a shrimp farm in Songkhla Province, Thailand. Larvae were acclimatized in a tank containing 5 l of 2% artificial sea water (ASW) with aeration at room temperature (30°C) and fed with a commercial diet twice a day. The experiment was divided into 6 groups: ASW (PL24) control, AHPND Vp, BALO control, and AHPND Vp interactions with BALO at final concen-

trations of 10^2, 10^4 and 10^6 PFU ml^{-1}. Briefly, PL24 were introduced into the tanks containing AHPND Vp at the final concentration of 10^7 CFU ml^{-1}, and various concentrations of BALO were added to the tanks after 15 min. In each group, 20 shrimp larvae were investigated and 2 replicates were performed. The mortality of larvae was recorded daily for 7 d.

Biofilm assay

To evaluate the ability of BALOs to remove biofilm formed by *V. parahaemolyticus*, the formation of biofilm by AHPND Vp and clinical and environmental *V. parahaemolyticus* isolates was assessed by quantitative determination (Nesper et al. 2001, Chanyi & Koval 2014). Briefly, 200 µl of an overnight culture of *V. parahaemolyticus* grown in LB broth supplemented with 2% NaCl were inoculated into 96-well microtiter plates and incubated at room temperature (30°C) for 24 h. The wells were washed 3 times with water to remove planktonic cells, and 200 µl of a BALO suspension (10^6 PFU ml^{-1}) in DNB were inoculated into the preformed *V. parahaemolyticus* biofilms and incubated at room temperature for 24 h. The wells were then washed 3 times and fixed with glutaraldehyde. The cells were stained with 200 µl of crystal violet (0.4% w/v) for 15 min, washed with water and dried, then destained with ethanol-acetone (80:20). The biofilm production was analyzed by color development using a microplate reader at a wavelength of 570 nm. Biofilm formation of either *V. parahaemolyticus* alone or a BALO strain alone including LB broth supplemented with 2% NaCl was used as a control. In all experiments, the results obtained were an average of 12 wells.

Statistical analysis

Statistical analysis was carried out using SPSS 11.5 software to observe differences in each experiment. All values were subjected to 1-way analysis of variance (ANOVA). The data are presented as the mean and standard deviation (SD) for the indicated numbers of each experiment. Means were separated using Duncan's test at $p < 0.05$. The differences observed were considered statistically significant at $p < 0.05$.

RESULTS

Isolation and identification of BALOs

In total, 25 samples were collected including 13 water and 12 sediment samples from Yor Island and shrimp farms. Three BALO strains were isolated from the water and 6 from the sediment samples (Table 1). Confirmation by 16S rRNA sequencing revealed that all of them were *Bacteriovorax* spp. with 89 to 100% homology to the reference strains in GenBank. A comparison of the phylogenetic tree analysis with 11 *Bacteriovorax* spp. reference strains in GenBank (see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d124p223_supp.pdf) indicated that *Bacteriovorax* NBV 2-5 were 89 to 99% similar in sequence to the NE1, DA5, DD1, and NB2 reference strains. *Bacterivorax* MBV 5-6 showed 100% homology to the RM2T4-S, RM3S1-S, RM2B2-S, RL1T4-S, RM3T4-S, and RM3S3-S reference strains, whereas *Bacterivorax* BV-A was 91% homologous to this group. *Bacterivorax* MBV 4 and NBV1 displayed around 94 to 100% similarity to BV-A and the rest of the *Bacteriovorax* obtained in this study (Table 1 and Fig. S1).

Table 1. Isolation of *Bdellovibrio* and like organisms (BALOs) from environmental samples and shrimp farms

Source	Sample type	No. of positive isolates	Isolate code	NCBI description	Homology (%)
Yor island	Water	3	MBV4	*Bacteriovorax* sp. B3S2-S	94
			MBV5	*Bacteriovorax* sp. RM3T4-S	100
			MBV6	*Bacteriovorax* sp. RM3T4-S	100
	Sediment	2	NBV1	*Bacteriovorax* sp. NE1	100
			NBV2	*Bacteriovorax* sp. NE1	89
Trang farm	Sediment	1	BV-A	*Bacteriovorax* sp. DA5	91
Samutsongkram farm	Sediment	2	NBV3	*Bacteriovorax* sp. NE1	99
			NBV4	*Bacteriovorax* sp. NE1	99
Songkhla farm	Sediment	1	NBV5	*Bacteriovorax* sp. NE1	98

Susceptibility of Gram-positive and Gram-negative bacteria to BALOs

The ability of 9 isolates of *Bacteriovorax* spp. to lyse Gram-positive and Gram-negative bacteria, including *Vibrio* spp., was evaluated. Two isolates of *Bacteriovorax* spp. could attack *Staphylococcus aureus*, and 4 could predate *E. coli*, whereas 5 to 6 isolates possessed the ability to lyse *Vibrio vulnificus*, *V. cholerae*, and *V. alginolyti-*

Table 2. Susceptibility of other bacteria (*Staphylococcus aureus*, *Escherichia coli*) and *Vibrio* spp. to *Bacteriovorax* spp. The experiment was performed independently 3 times; numbers shown are the numbers of positive experiments out of 3; dashes indicate that all 3 experiments were negative. Vv: *Vibrio vulnificus*; Vc: *V. cholerae*; Va: *V. alginolyticus*; Vp: *V. parahaemolyticus*; AHPND Vp: acute hepatopancreatic necrosis disease-causing *V. parahaemolyticus*

Bacteriovorax isolates	Hosts													
	Other bacteria		*Vibrio* spp.			AHPND Vp					Vp			
											Clinical		Environmental	
	S. aureus	*E. coli*	Vv	Vc	Va	EMS_1S_2	VP12	7.2L3	PeP_{16}	6.1L3	PSU5666	PSU5668	PSU5147	PSU5150
BV-A	+3	+3	+3	+3	+3	+3	+3	+3	+3	+3	+3	–	–	+3
MBV4	–	–	+2	–	–	+3	+3	+3	+3	+3	+3	–	+3	+3
MBV5	+3	–	–	+2	+2	+3	+3	+3	+3	+3	–	–	–	–
MBV6	–	–	–	–	–	+3	+3	+3	+3	+3	–	–	–	–
NBV1	–	–	+2	–	+3	+3	+3	+3	+3	+3	–	–	–	–
NBV2	–	+2	+3	+3	+3	+3	+3	+3	+3	+3	+3	–	–	–
NBV3	–	+3	+3	+3	+2	+3	+3	+3	+3	+3	+3	+3	+3	+1
NBV4	–	+3	–	+2	+3	+3	+3	+3	+3	+3	+3	–	–	–
NBV5	–	–	–	+3	–	+3	+3	+3	+3	+3	–	+1	–	–
Total	2	4	5	6	6	9	9	9	9	9	5	2	2	3

cus (Table 2). All of them could lyse all 5 AHPND Vp tested isolates; however, only 2 to 5 isolates of *Bacteriovorax* spp. could attack clinical and environmental *V. parahaemolyticus*.

Factors involved in interaction between a BALO strain and AHPND Vp

To investigate the optimal ratio for the interaction of BALOs with AHPND Vp, *Bacteriovorax* BV-A was selected because it was isolated from a shrimp farm that was less severely affected by AHPND. In addition, it was able to attack many bacterial strains (Table 2). BV-A was incubated with AHPND Vp PSU 5429 at a ratio between 1:10 and 1:10 000. Although the numbers of AHPND Vp continuously decreased in every interaction ratio after 1 d of incubation, the highest reduction in numbers of AHPND Vp (91.1%) was detected at a ratio of 1:10 (Fig. 1). Thus, this ratio was used for interaction evaluation between these organisms throughout this study.

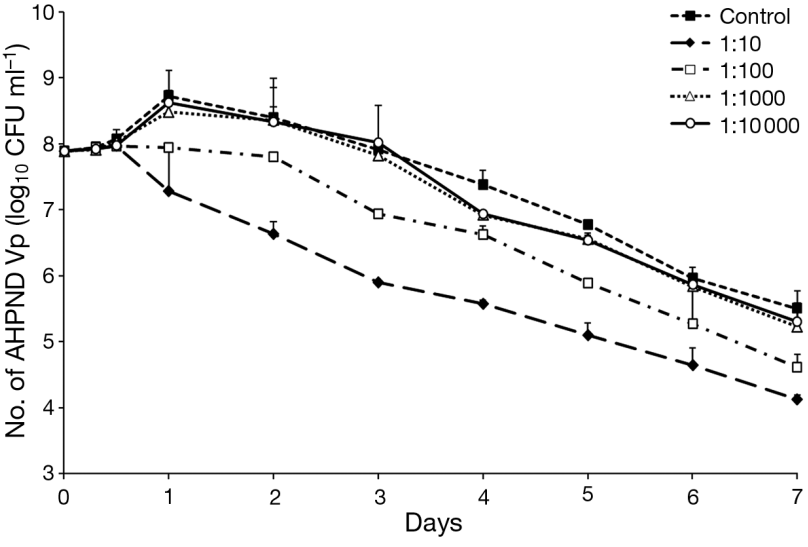

Fig. 1. Determination of the optimal ratio for interaction between *Bacteriovorax* BV-A and acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* (AHPND Vp). BV-A was incubated with AHPND Vp at ratios between 1:10 and 1:10 000 for 7 d, and the numbers of AHPND Vp were enumerated daily. The control contained only AHPND Vp. Values are mean ± SD of 2 experiments; each experiment was performed in triplicate. The highest reduction in numbers of AHPND Vp (91.1%) was detected at a ratio of 1:10

Reduction in numbers of AHPND Vp in co-culture between BV-A and AHPND Vp in the whole period of time was greater at 30°C than at 25 or 37°C (Fig. S2A in the Supplement). In addition, 90.8 to 95.3% reduction in numbers of AHPND Vp was detected in the co-culture at NaCl concentrations between 1 and 3% (Fig. S2B). No significant difference in the reduction of AHPND Vp isolate was observed after co-cultivation with BV-A at pH between 7 and 8 (Fig. S2C). In Thailand, the level of salinity in shrimp farms is between 1 and 3% (Flaherty et al. 2000), and the ambient temperature is 30°C. Therefore, 2% NaCl and 30°C were applied for subsequent co-culture of *Bacteriovorax* and AHPND Vp in DNB at pH 7.6. This pH was selected because the typical pH of seawater lies between 7.5 and 8.4, and the appropriate pH of

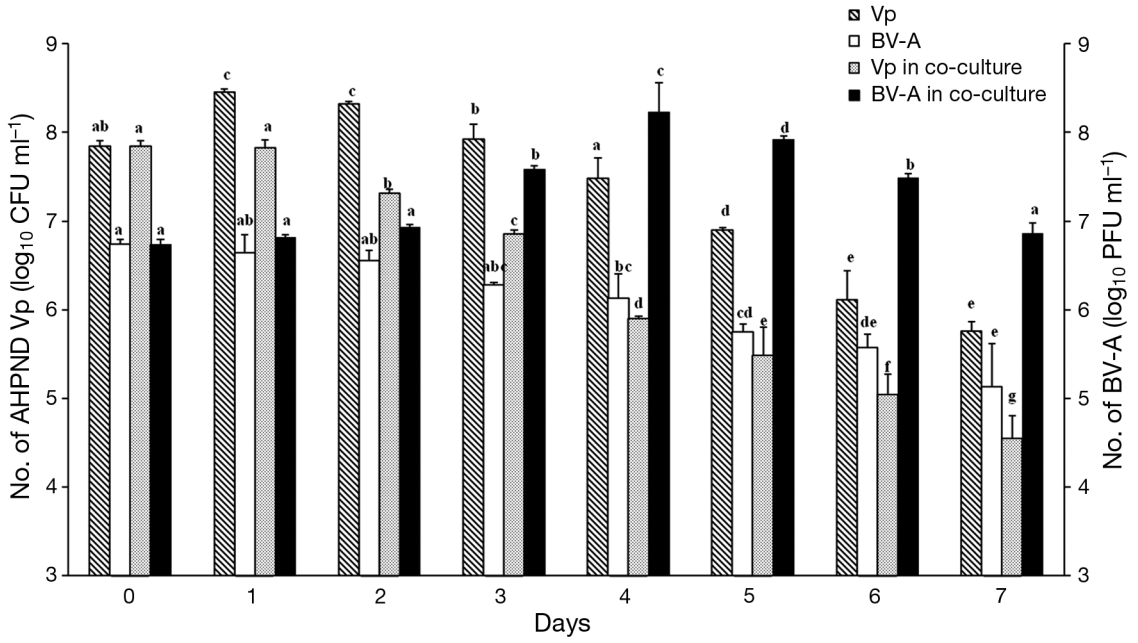

Fig. 2. Potential of *Bacteriovorax* BV-A to inhibit acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* (AHPND Vp) *in vitro*. BV-A was co-cultured with AHPND Vp for 7 d, and each organism was enumerated daily and compared to the BV-A and AHPND Vp controls. Values are mean ± SD of 2 experiments. Different letters above bars show values that are significantly different ($p < 0.05$). Each value is compared within each category

water in shrimp ponds should be maintained between 7.5 and 8.5 (Anh et al. 2010).

In vitro interaction between a BALO strain and AHPND Vp

One milliliter each of BV-A (10^6 PFU ml^{-1}) and AHPND Vp PSU 5429 (10^7 CFU ml^{-1}) were mixed in 100 ml of DNB containing 2% NaCl, pH 7.6, and incubated at 30°C. After 2 d of incubation, the numbers of AHPND Vp were significantly lower than in the AHPND Vp control and continuously decreased over the following days (Fig. 2). However, the numbers of BV-A significantly increased after 3 d of incubation and continued to do so until Day 5 of incubation, before decreasing until Day 7. In the AHPND Vp control group, the bacterial numbers significantly increased during Day 1 and 3 of incubation and subsequently decreased from Days 5 to 7, whereas a slight decrease in numbers of the BV-A control was detected on Day 1 and carried on to Day 7.

In vivo reduction of shrimp larval mortality by BV-A

Shrimp post-larvae (PL24) were inoculated with AHPND Vp PSU 5429 at a final concentration of 10^7 CFU ml^{-1}, and various final concentrations of BV-A between 10^2 and 10^6 PFU ml^{-1} were added into the shrimp tanks. The mortality of the post-larvae was determined daily for 7 d. On Day 1, approximately 7.5, 2.5, 2.5, and 0% of PL24 were dead in the AHPND Vp control and in the infected post-larval groups treated with 10^2, 10^4, and 10^6 PFU ml^{-1} BV-A, respectively; dead post-larvae in those groups increased to 50.0, 30.0, 20.0, and 17.5% on Day 3 (Fig. 3). At the end of treatment, more than 90% of post-larvae were dead in the AHPND Vp control, whereas in the infected groups containing BV-A at final concentrations of 10^2, 10^4, and 10^6 PFU ml^{-1}, mortality was 72.5, 62.5, and 47.5%, respectively. No post-larvae died in the post-larvae and BV-A control groups.

Reducing *V. parahaemolyticus* biofilm formation by BV-A

AHPND Vp and clinical and environmental *V. parahaemolyticus* isolate biofilms were pre-formed in 96-well microtiter plates for 24 h, the planktonic cells were removed, and the remaining biofilms were evaluated after adding BV-A and further incubated for 24 h. Biofilm formation of the 3 categories of *V. parahaemolyticus* was significantly reduced by BV-A (Fig. 4).

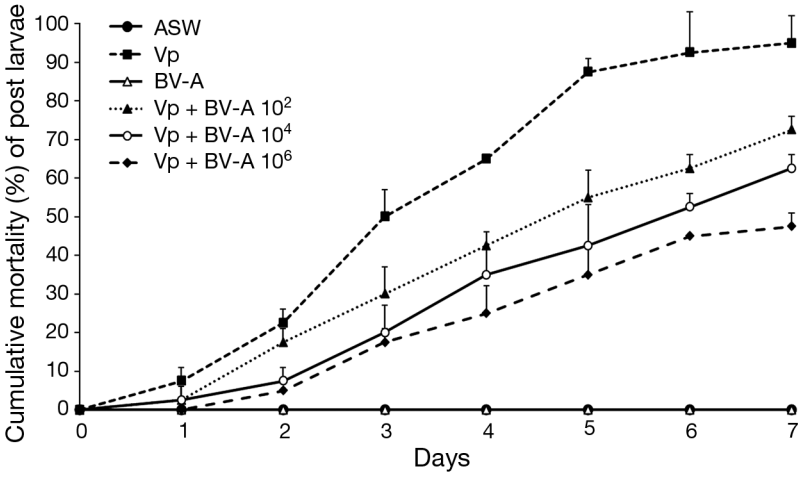

Fig. 3. Efficacy of *Bacteriovorax* BV-A to decrease shrimp post-larval mortality after treatment with acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* (AHPND Vp). *Litopenaeus vannamei* post-larvae (PL24) were exposed to AHPND Vp and subsequently treated with *Bacteriovorax* BV-A at concentrations of 10^2, 10^4, and 10^6 plaque-forming units (PFU) ml^{-1}. Dead post-larvae were counted daily. PL24 treated with artificial seawater (ASW), AHPND Vp (10^7 CFU ml^{-1}), or BV-A (10^6 PFU ml^{-1}) alone were used as controls

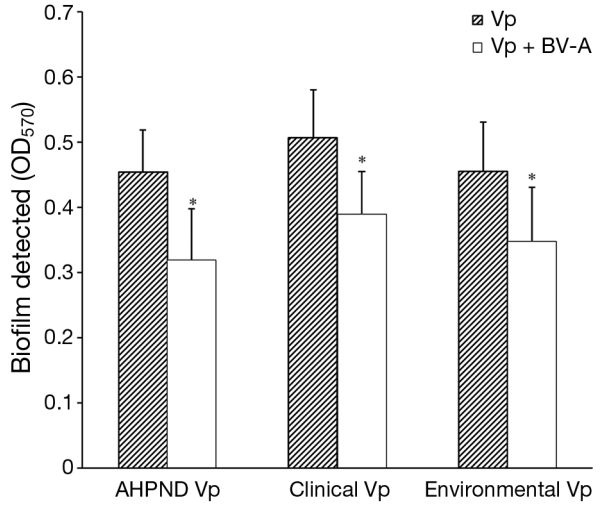

Fig. 4. Capability of *Bacteriovorax* BV-A to decrease biofilm formation of acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* (AHPND Vp) and clinical and environmental isolates of *V. parahaemolyticus*. *V. parahaemolyticus* biofilms were pre-formed in 96-well microtiter plates for 24 h, and BV-A suspension was added after removal of planktonic cells. Biofilm was quantitated using crystal violet staining. * indicates significant differences ($p < 0.05$)

DISCUSSION

AHPND has caused an economic crisis in shrimp aquaculture. BALOs are ubiquitously distributed in the environment and target many prey bacteria. This makes them an attractive candidate for control of *Vibrio parahaemolyticus*, the causative agent of AHPND. In this work, various samples were obtained from Yor Island and infected shrimp farms. This island was selected because it is close to many shrimp farms located on the peninsular coast, and BALOs prefer to attack bacteria from the same environment (Pineiro et al. 2004). The organisms were more predominant in sediment than in water; 6 out of 12 sediments were positive (50%), but they were only detected in 3 out of 13 water samples (23%; Table 1). 16S rRNA sequencing was performed for identification of all BALOs obtained in this study without any further identification; therefore, the isolates were specified as the genus of their closest BLAST homology. Although all of the isolates collected from Yor Island were identified as *Bacteriovorax* spp., phylogenetic analysis differentiated 2 of them (MBV4 and NBV1) with 100% confidence because they formed an independent branch (Fig. S1). Previously, there had been no evidence that Gram-positive bacteria were susceptible to BALOs; recently, however, *Bdellovibrio bacteriovorus* HD 100 has been demonstrated to decrease numbers of *Staphylococcus aureus* in broth culture (Iebba et al. 2014). In the present study, only 2 out of 9 *Bacteriovorax* spp. (BV-A and MBV-5) were able to attack *S. aureus* (Table 2). However, all of them could lyse all AHPND Vp tested isolates, whereas their capabilities to attack clinical and environmental *V. parahaemolyticus* were lower. This may be because most of the *Bacteriovorax* spp. were isolated from AHPND-affected shrimp ponds, and AHPND Vp PSU 5429 was used as a host for propagation. In this work, although MBV-5 and MBV-6 showed 100% homology to *Bacteriovorax* RM3S3-S including the other 6 *Bacteriovorax* reference strains (Fig. S1), their bacteriolytic activities toward prey bacteria were slightly different (Table 2). The information of 16S sequencing might not be enough to differentiate some BALO strains. The gene encoding the β-subunit of RNA polymerase (*rpoB*) is more discriminating than the 16S rRNA gene for differentiation of saltwater members of the genus *Bacteriovorax* (Pineiro et al. 2008). Additionally, the mechanism of BALOs to select their prey is not clearly understood, and variability in prey range between 2 closely related BALO strains has been documented (Chanyi et al. 2013).

The optimal ratio for interaction between *Bacteriovorax* BV-A and AHPND Vp was evaluated. In this work, AHPND Vp PSU 5429 at a concentration of 10^7 CFU ml^{-1} was selected for the assay because investigation of AHPND in a shrimp farm in Mexico revealed that different virulence of *V. parahaemolyticus* depended on bacterial density, and the minimum infective density was 10^4 CFU ml^{-1} (Soto-Rodriguez et al. 2015). In addition, no shrimp mortality was observed at densities below this value, and less virulent strains did not induce 100% mortality. In our study, the reduction of AHPND Vp isolates was highest at a ratio of 1:10 between BV-A and AHPND Vp. Thus, this ratio was used for the consecutive assays.

To evaluate the capability of *Bacteriovorax* BV-A to decrease numbers of AHPND Vp *in vitro*, co-culture of those organisms was conducted. Each of AHPND Vp and BV-A was included for comparison. We found that at the end of incubation (Day 7), the numbers of both control organisms had decreased (Fig. 2). This might be due to the depletion of nutrients in DNB as the growth of AHPND Vp approached the decline phase, whereas reduction of BV-A was due to the lack of prey. In co-culture, the numbers of AHPND Vp decreased significantly more than that of the control group, indicating the potential of BV-A to eliminate this bacterium. This was clearly apparent on Day 2 of incubation, and the numbers of BV-A started increasing in the subsequent days. On Day 5, the numbers of BV-A in co-culture started decreasing, and this continued until the end of the incubation period, suggesting that this decline might be due to the decrease in numbers of prey.

To evaluate the effectiveness of BALOs in the prevention of AHPND Vp infection in shrimp, a different concentration of BV-A was applied to AHPND Vp-infected PL24. Post-larval mortality was decreased after treatment with the high concentration of BV-A (Fig. 3). This indicates the protective efficacy of BALO for post-larval shrimp. However, in this study, the mortality of post-larvae was reduced by approximately 50% with a 1:10 ratio of BV-A to AHPND Vp. Therefore, to improve the effectiveness of the treatment, a higher concentration of BALO is suggested.

V. parahaemolyticus can form biofilms on shrimp surfaces (Han et al. 2016). Investigation of 35 AHPND Vp isolates derived from shrimp farms in Mexico revealed that all of them were able to form moderate to strong biofilms (López-Leónl et al. 2016). In shrimp farming, the molting process of shrimp causes an accumulation of bacterial biofilms at the bottom of the pond. Iebba et al. (2014) reported that BALOs could target their prey both in water and in biofilm. In the current work, we demonstrated the potential of BV-A to significantly diminish biofilm formed by AHPND Vp and by clinical and environmental *V. parahaemolyticus* isolates (Fig. 4). Therefore, this evidence supports the advantage of using *Bacteriovorax* as a biocontrol in shrimp aquaculture.

BALOs have a wide prey range, but the way they select their prey is not well understood (Rogosky et al. 2006, Chen et al. 2011). *Bdellovibrio* directs itself towards its prey by flagellar motility and chemotactic responses (Straley & Conti 1977, Lambert et al. 2003). Pilus fibers may also be involved in the early stage of predation. Three *pilA* mutants of *Bdellovibrio bacteriovorus* were unable to attack their prey compared with the wild type (Evans et al. 2007). In addition, Bd0112 (homologue to *pilQ*) and Bd3852 (homologue to 1 of 2 *pilT*) are implicated in *B. bacteriovorus* predation (Medina et al. 2008). BALOs generally possess a periplasmic life cycle; however, recently, an epibiotic life cycle has been demonstrated in the Gram-negative bacteria *Acinetobacter*, *Aeromonas*, *Caulobacter*, and *Delftia* (Chanyi et al. 2013). In the epibiotic life cycle, predators attach to the prey surface and perform binary fission on the outer surface of the prey cell while prey cytoplasmic contents decrease. Iebba et al. (2014) demonstrated that *B. bacteriovorus* HD 100 attacked *S. aureus* in the epibiotic style. In addition, Monnappa et al. (2014) reported extracellular protease released from *B. bacteriovorus* HD 100 to degrade *S. aureus* biofilm and reduce its virulence. We do not know which life cycle(s) *Bacteriovorax* BV-A obtained in this work uses for attacking AHPND Vp. It would be of interest to determine the attack mechanism of this predator in the future.

In conclusion, this work demonstrates the potential of BALOs to control AHPND Vp in shrimp farms. One genus of BALOs (*Bacteriovorax*) was predominantly isolated from water and sediment samples. The optimal ratio of *Bacteriovorax* BV-A to interact with AHPND Vp was 1:10. *In vitro*, BV-A decreased the numbers of AHPND Vp within 2 d of the co-culture, and *in vivo*, it reduced mortality of post-larvae infected with AHPND Vp by around 50%. This indicates the preventive efficiency of BALOs in the control of AHPND Vp in shrimp aquaculture.

Acknowledgements. This work was supported in part by a subcontract from grant A27134 from CPF (Thailand) awarded to Harvard University and in part by the Royal Golden Jubilee PhD Program, Thailand Research Fund (Grant No. PHD/0213/2556).

LITERATURE CITED

Anh PT, Kroeze C, Bush SR, Mol APJ (2010) Water pollution by intensive brackish shrimp farming in south-east Vietnam: causes and options for control. Agric Water Manag 97:872–882

Cao H, He S, Wang H, Hou S, Lu L, Yang X (2012) Bdellovibrios, potential biocontrol bacteria against pathogenic *Aeromonas hydrophila*. Vet Microbiol 154:413–418

Chanyi RM, Koval SF (2014) Role of Type IV pili in predation by *Bdellovibrio bacteriovorus*. PLOS ONE 9:e113404

Chanyi RM, Ward C, Pechey A, Koval SF (2013) To invade or not to invade: two approaches to a prokaryotic predatory life cycle. Can J Microbiol 59:273–279

Chen H, Athar R, Zheng G, Williams HN (2011) Prey bacteria shape the community structure of their predators. ISME J 5:1314–1322

Chu WH, Zhu W (2010) Isolation of *Bdellovibrio* as biological therapeutic agents used for the treatment of *Aeromonas hydrophila* infection in fish. Zoonoses Public Health 57: 258–264

Dashiff A, Junka RA, Libera M, Kadouri DE (2011) Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*. J Appl Microbiol 110:431–444

Davidov Y, Friedjung A, Jurkevitch E (2006) Structure analysis of a soil community of predatory bacteria using culture-dependent and culture independent methods reveals a hitherto undetected diversity of *Bdellovibrio*-and-like organisms. Environ Microbiol 8:1667–1673

Evans KJ, Lambert C, Sockett RE (2007) Predation by *Bdellovibrio bacteriovorus* HD100 requires type IV pili. J Bacteriol 189:4850–4859

Flaherty M, Szuster B, Miller P (2000) Low salinity inland shrimp farming in Thailand. Ambio 29:174–179

Fratamico PM, Cooke PH (1996) Isolation of Bdellovibrios that prey on *Escherichia coli* O157:H7 and *Salmonella* species and application for removal of prey from stainless steel surfaces. J Food Saf 16:161–173

Han N, Mizan MFR, Jahid IK, Ha SD (2016) Biofilm formation by *Vibrio parahaemolyticus* on food and food contact surfaces increases with rise in temperature. Food Control 70:161–166

Hong XP, Xu D, Zhuo Y, Liu HQ, Lu LQ (2016) Identification and pathogenicity of *Vibrio parahaemolyticus* isolates and immune responses of *Penaeus* (*Litopenaeus*) *vannamei* (Boone). J Fish Dis 39:1085–1097

Iebba V, Totino V, Santangelo F, Gagliardi A and others (2014) *Bdellovibrio bacteriovorus* directly attacks *Pseudomonas aeruginosa* and *Staphylococcus aureus* cystic fibrosis isolates. Front Microbiol 5:280

Joshi J, Srisala J, Truong VH, Chen IT and others (2014) Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). Aquaculture 428-429:297–302

Jurkevitch E, Minz D, Ramati B, Barel G (2000) Prey range characterization, ribotyping, and diversity of soil and rhizosphere *Bdellovibrio* spp. isolated on phytopathogenic bacteria. Appl Environ Microbiol 66:2365–2371

Kongrueng J, Yingkajorn M, Bunpa S, Sermwittayawong N, Singkhamanan N, Vuddhakul V (2015) Characterization of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease in southern Thailand. J Fish Dis 38:957–966

Lai HC, Ng TH, Ando M, Lee CT and others (2015) Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in shrimp. Fish Shellfish Immunol 47:1006–1014

Lambert C, Smith MCM, Sockett RE (2003) A novel assay to monitor predator-prey interactions for *Bdellovibrio bacteriovorus* 109J reveals a role for methyl-accepting chemotaxis proteins in predation. Environ Microbiol 5: 127–132

Li H, Liu C, Chen L, Zhang X, Cai J (2011) Biological characterization of two marine *Bdellovibrio*-and-like organisms isolated from Daya bay of Shenzhen, China and their application in the elimination of *Vibrio parahaemolyticus* in oyster. Int J Food Microbiol 151:36–43

Li H, Chen C, Sun Q, Liu R, Cai J (2014) *Bdellovibrio* and like organisms enhanced growth and survival of *Penaeus monodon* and altered bacterial community structures in its rearing water. Appl Environ Microbiol 80:6346–6354

López-Leónl P, Luna-González A, Escamilla-Montes R, Flores-Miranda MC, Fierro-Coronadol JA, Álvarez-Ruiz P, Diarte-Plata G (2016) Isolation and characterization of infectious *Vibrio parahaemolyticus*, the causative agent of AHPND, from the whiteleg shrimp (*Litopenaeus vannamei*). Lat Am J Aquat Res 44:470–479

Medina AA, Shanks RM, Kadouri DE (2008) Development of a novel system for isolating genes involved in predator-prey interactions using host independent derivatives of *Bdellovibrio bacteriovorus* 109J. BMC Microbiol 8:33

Monnappa AK, Dwidar M, Seo JK, Hur JH, Mitchell RJ (2014) *Bdellovibrio bacteriovorus* inhibits *Staphylococcus aureus* biofilm formation and invasion into human epithelial cells. Sci Rep 4:3811

Nesper J, Lauriano CM, Klose KE, Kapfhammer D, Kraiss A, Reidl J (2001) Characterization of *Vibrio cholerae* O1 El tor *galU* and *galE* mutants: influence on lipopolysaccharide structure, colonization, and biofilm formation. Infect Immun 69:435–445

Nunan L, Lightner D, Pantoja C, Gomez-Jimenez S (2014) Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. Dis Aquat Org 111:81–86

Pineiro SA, Sahaniuk GE, Romberg E, Williams HN (2004) Predation pattern and phylogenetic analysis of *Bdellovibrionacea* from the Great Salt Lake, Utah. Curr Microbiol 48:113–117

Pineiro SA, Williams HN, Stine OC (2008) Phylogenetic relationships amongst the saltwater members of the genus *Bacteriovorax* using *rpoB* sequences and reclassification of *Bacteriovorax stolpii* as *Bacteriolyticum stolpii* gen. nov., comb. nov. Int J Syst Evol Microbiol 58:1203–1209

Rogosky AM, Moak PL, Emmert EA (2006) Differential predation by *Bdellovibrio bacteriovorus* 109J. Curr Microbiol 52:81–85

Sockett RE, Lambert C (2004) *Bdellovibrio* as therapeutic agents: a predatory renaissance? Nat Rev Microbiol 2: 669–675

Soto-Rodriguez SA, Gomez-Gil B, Lozano-Olvera R, Betancourt-Lozano M, Morales-Covarrubias MS (2015) Field and experimental evidence of *Vibrio parahaemolyticus* as the causative agent of acute hepatopancreatic necrosis disease of cultured shrimp (*Litopenaeus vannamei*) in Northwestern Mexico. Appl Environ Microbiol 81: 1689–1699

Starr MP (1975) *Bdellovibrio* as symbiont; the associations of Bdellovibrios with other bacteria interpreted in terms of a generalized scheme for classifying organismic associations. Symp Soc Exp Biol 29:93–124

Straley SC, Conti SF (1977) Chemotaxis by *Bdellovibrio bacteriovorus* toward prey. J Bacteriol 132:628–640

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10: 512–526

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729

Tran L, Nunan L, Redman RM, Mohney LL, Pantoja CR, Fitzsimmons K, Lightner DV (2013) Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis Aquat Org 105:45–55

Williams HN, Pineiro SA (2006) Ecology of the predatory *Bdellovibrio* and like organisms. Microbiol Monogr 4: 213–248

Yang YT, Chen IT, Lee CT, Chen CY and others (2014) Draft genome sequences of four strains of *Vibrio parahaemolyticus*, three of which cause early mortality syndrome/acute hepatopancreatic necrosis disease in shrimp in China and Thailand. Genome Announc 2:e00816–14

Editorial responsibility: Jeffrey Shields, Gloucester Point, Virginia, USA

Submitted: October 4, 2016; Accepted: March 6, 2017
Proofs received from author(s): April 14, 2017