ORIGINAL ARTICLE

Selection of proteolytic bacteria with ability to inhibit *Vibrio harveyi* during white shrimp (*Litopenaeus vannamei*) cultivation

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Abstract

Rattanachuay, P., Kantachote, D. and Suntinanalert, P.
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Songklanakarin J. Sci. Technol., 2007, 29(2) : 235-243

Five isolates of bacteria with high proteolytic activity, isolated from water samples of intensive shrimp ponds in southern Thailand, were selected to test for the ability to control the shrimp pathogen *Vibrio harveyi*. 70 µl of each culture broth were investigated for their ability to inhibit *V. harveyi* using an agar well diffusion test but only one isolate W3 gave a reasonable sized inhibition zone of 21.62 mm. This zone was similar to that of oxolinic acid (2 µg) and sulfamethoxazole (25 µg). The W3 isolate was identified as *Pseudomonas* sp. Shrimp cultivation in aquaria was conducted to investigate the inhibition of *V. harveyi* by the isolate W3. The experiment consisted of a treatment of the shrimp culture with an inoculum of the isolate W3 and *V. harveyi* (biocontrol set), a positive control set (only inoculation of *V. harveyi*) and a negative control set as without inoculation. No mortality was found in the negative control. Shrimp mortality in the

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Received, 13 June 2006 Accepted, 11 October 2007

biocontrol set (33%) was lower than that in the positive control set (40%); however, it showed no significant difference (p>0.05). The average numbers of *V. harveyi* over 12 days of the biocontrol set were lower than those in the positive control set by about 1 log cycle although the numbers were not significantly different (p>0.05). The shrimp growth rate at day 32 of cultivation was in order of the biocontrol treatment (10.17%) > the negative control treatment (9.44%) > the positive control set (9.28%), but no significant difference (p>0.05) was observed among treatments.

Key words : proteolytic bacteria, *Vibrio harveyi*, inhibition, *Pseudomonas* sp. W3, white shrimp cultivation

บทคัดย่อ

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การคัดเลือกแบคทีเรียย่อยโปรตีนที่มีความสามารถในการยับยั้ง *Vibrio harveyi* ในการเพาะเลี้ยงกุ้งขาว (*Litopenaeus vannamei*)

ว. สงขลานครินทร์ วทท. 2550 29(2) : 235-243

การคัดเลือกแบคทีเรียที่สามารถย่อยโปรตีนได้ดีจำนวน 5 ไอโซเลท ซึ่งแยกได้จากน้ำบ่อเลี้ยงกุ้งแบบพัฒนาในภาคใต้ของประเทศไทยเพื่อควบคุม *Vibrio harveyi* ซึ่งเป็นแบคทีเรียที่ก่อโรคในกุ้ง ทดสอบการยับยั้งด้วยวิธี Agar well diffusion โดยใช้น้ำเลี้ยงเชื้อ 70 µl พบว่ามีเพียงไอโซเลท W3 ที่ให้วงใสการยับยั้งเท่ากับ 21.62 มม. ซึ่งผลการยับยั้งใกล้เคียงกับยาปฏิชีวนะ 2 ชนิด คือ αolinic acid (2 µg) และ sulfamethoxazole (25 µg) และจากการเทียบเคียงแบคทีเรียไอโซเลท W3 พบว่าเป็น *Pseudomonas* sp. เพื่อที่จะตรวจสอบการยับยั้งแบคทีเรียก่อโรคกุ้ง *V. harveyi* ของแบคทีเรียที่คัดเลือกได้ จึงได้จำลองภาวะการเลี้ยงกุ้งในตู้กระจกโดยประกอบด้วยชุดทดลองที่เติมแบคทีเรียทั้ง 2 ชนิด (ไอโซเลท W3 และ *V. harveyi*) ชุดควบคุมทางบวกที่เติมเฉพาะ *V. harveyi* และชุดควบคุมทางลบที่ไม่ได้เติมแบคทีเรีย ไม่พบการตายของกุ้งในชุดควบคุมทางลบ แต่พบการตายในชุดที่เติมแบคทีเรียทั้ง 2 ชนิด (33%) ซึ่งต่ำกว่าในชุดควบคุมทางบวก (40%) แต่ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ (p>0.05) นอกจากนี้ยังพบว่าในชุดที่เติมทั้งไอโซเลท W3 และ *V. harveyi* หลัง 12 วันของการเลี้ยง พบจำนวน *V. harveyi* น้อยกว่าในชุดควบคุมทางบวกประมาณ 1 log cycle แม้ว่าไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ (p>0.05) และสำหรับอัตราการเจริญทางบวกของกุ้งที่ 32 วันในชุดที่เติมเชื้อทั้ง 2 ชนิด (10.17%) สูงกว่าชุดควบคุมทางลบ (9.44%) และในชุดควบคุมทางบวก (9.28%) แต่ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ (p>0.05)

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Thailand has been one of the world's leading countries for shrimp production (Silva, 1998; Holmström *et al*., 2003). However, shrimp production per crop has decreased since the early 2000s due to problems of the quality of broodstocks and outbreaks of viral and bacterial infections. *Vibrio harveyi*, the cause of vibriosis (Chythanya *et al*., 2002), is the most common pathogenic bacteria of cultured shrimp that can cause high mortalities in the larvaculture and growout phases of crustacean aquaculture (Gomez-Gil *et al*., 2000; Vijayan *et al*., 2006). In the past, shrimp farmers widely used antibiotics to promote shrimp growth and to treat shrimp diseases (Holmström *et al*., 2003). The use of antibiotics during shrimp cultivation has led to problems of bacterial drug resistance (Graslund *et al*., 2002). In 2002 the EU community rejected exported Thai shrimp that was contaminated with chloramphenicol and nitrofuran. As a consequence, the Thai Department of Fisheries has developed a set of guidelines for a Code of Conduct and farm inspections in order to comply

with Good Aquaculture Practices to restrict antibiotic residues to ensure the quality and hygiene of Thai shrimps bound for export. (http://www.fisheries.go.th, 2003). In addition, the Ministry of Public Health in the year 2004 gave the award of best "Food Safety slogan" to the safe shrimp for human consumption campaign.

It has long been recognized that shrimp production is affected by 3 main factors, namely: shrimp health, pathogens and environment concerns of water and sediment quality. Bacteria with anti-Vibrio activity have potential application as biocontrol agents and their use would be an attractive way to avoid antibiotics during shrimp culture, with no harm to the environment. Furthermore, probiotics have gained public acceptance as being more effective than administering antibiotics or chemical substances. A variety of microbes have been investigated to use as probiotics in aquaculture such as Gram positive, Gram negative bacteria, yeast and unicellular algae (Irianto and Austin, 2002; Vijayan *et al*., 2006). Moreover, there are several microbial products marketed for aquaculture use to clean up the pond and maintain good water quality in intensive shrimp ponds (Gatesoupe, 1999). Shrimp feed contains a high amount of protein, thereby in shrimp ponds proteolytic bacteria are likely to be one of the bacterial groups that will govern the quality of water and sediment in shrimp ponds, and have a major influence on shrimp health. Proteolytic bacteria with ability to inhibit shrimp pathogenic *Vibrio harveyi* would be interesting to use as the concomitant bi-function in shrimp cultivation by improving water quality and controlling shrimp disease. In this paper, only anti-Vibrio activity of proteolytic bacteria is presented. Therefore, the objective of this work was to select a proteolytic bacterium with anti-Vibrio activity for use as an agent for the biocontrol of *V. harveyi* during shrimp cultivation.

Materials and methods

1. Bacteria

The following proteolytic bacteria; W3, W6, W10, W30 and W310, were isolated from water samples, which collected from various intensively farmed shrimp ponds in Pattani and Songkhla provinces. They were selected based on their protease activity using degree of hydrolysis and protease activity (Rattanachuay *et al*., 2005). They were investigated for their ability to inhibit the shrimp pathogen, *Vibrio harveyi*. The proteolytic bacteria were maintained and grown on Frazier gelatin medium (FGM) (Frazier and Rupp, 1982).

V. harveyi was supplied by Assoc. Prof. Dr. Varaporn Vuttakul, Department of Microbiology, Faculty of Science, Prince of Songkla University. *V. harveyi* was maintained by routine culture on Tryptic soy agar (TSA+1.5% NaCl) and Tryptic soy broth (TSB+1.5% NaCl), while TCBS agar (Thiosulfate citrate bile salt sucrose) was used to count *V. harveyi* during shrimp cultivation.

2. Inhibition of *Vibrio harveyi* by proteolytic bacteria

The agar well diffusion method (Chythanya *et al*., 2002) was used to test for any anti-Vibrio activity of proteolytic bacteria. Stock cultures of *V. harveyi*, were subcultured at least twice (18 h, 30°C) on fresh TSB plus 1.5% NaCl to ensure they were actively growing. The culture broth was adjusted to 0.5 McFarland standard ($1.5x10^8$ CFU/ml) and then swabbed over the surface of a TSA (1.5% NaCl) plate. Swabbing was then repeated to ensure an even distribution of inoculums. Wells (diameter 7 mm) were punched in the TSA plate and 70 µl of an 18 h culture (Chythanya *et al*., 2002) of the isolated W3, W6, W10, W30 and W3 10 in FGM (2% NaCl) were transferred into the wells and the plates were incubated at 30°C for 24 h. The antibiotic discs used as positive controls were: oxonilic acid (2 µg), norfloxacin (10 µg), tetracycline (30 µg) and sulphamethoxazole (25 µg). The diameters of the clear zones (inhibition zones) were measured using a Vernier caliper. The experiment was carried out in six replicates.

3. Characterization of an anti-Vibrio proteolytic bacterium

A proteolytic bacterium selected for its anti-

Vibrio activity was identified using biochemical and physiological tests according to Bergey's Manual of Systematic Bacteriology vol.1 (Krieg and Holt, 1984) and the test kit: API 20E (Biomerieux). In addition, 16S rDNA analysis was conducted by KU-Vector (KU-VECTOR CUSTOM DNA SYNTHESIS SERVICE Public-Private Technology Development & Transfer Center Central Research Center Building 8th Floor, Kasetsart University) to identify the bacterium (Alignment and Phylogenic tree by the DNASIS V 3.7 program). Scanning electron microscope (SEM) was conducted by the Scientific Equipment Center (SEC), Prince of Songkla University, to observe shape and arrangement of bacterial cells and flagella. Antibiotic sensitivity and haemolytic tests were also conducted. The following antibiotics 30 µg chloramphenicol, 10µg norfloxacin, 25 µg trimethoprim-sulfamethoxazole, 30 µg cephalothin, 30 µg tetracycline, 30 µg netilmicin, 30 µg amikacin and 10 µg gentamicin, that are normaly used to control *Pseudomonas* spp. were used. Blood agar was used to test haemolysis of the selected isolate.

4. Testing bacteria with antivibrio activity to protect shrimp from *V. harveyi* infection

4.1 Preparation of the test bacterium inoculum

The selected proteolytic bacterium with ability to inhibit *V. harveyi* was grown in FGM broth (plus 2% NaCl, pH 7) under optimal condition (150 rpm, 30°C) (Rattanachuay, 2005) until cells reached the late log phase. Cells in the late log phase were used as inoculants because they better resist changes to their environments (Vandenhove *et al*., 1991; Talaro and Talaro, 1996). Bacterial cells were centrifuged at 6000 rpm for 15 min and washed twice with normal saline solution and cell pellets were resuspended in the shrimp aquarium to obtain a final concentration at $1x10^6$ CFU/ml. This number ($1x10^6$ CFU/ml) was designed in this study due to consideration of economical reason in application. The *V. harveyi* inoculum was prepared by a similar procedure but using TSB plus 1.5% NaCl as the culture medium and also to obtain a final concentration as similar as with antagonist inoculants.

4.2 Preparation of the shrimp aquarium

Sea water with a salinity of 30 g/l and alkalinity of 100 mg/l was used. The sea water was sterilized with 30 mg/l chlorine followed by aeration with sterile air for 2 days to eliminate chlorine. The sterile water was added to an aquarium (45x45x60 cm) to the three quarters full level and then a nylon net was placed in each aquarium for shrimp attachment.

4.3 Shrimp cultivation

Ten, P30 white shrimps (*Litopenaeus vannamei*) from a shrimp hatchery in Trang province were released into each aquarium and fed 4 times/day at 7.00 am, 12.00 noon, 5.00 pm and 11.00 pm with 0.5 g (of commercial shrimp feed) each time. The shrimp aquaria were aerated for 24 h/day. Unfortunately the power supply failed at day 48, so the results of the experiment are presented only until 32 days of cultivation.

4.4 Experimental design

The ability of the selected proteolytic bacterium to act as a possible biological control agent against the shrimp pathogen *V. harveyi*, was conducted as follows. No addition of bacterial inoculum was used as a negative control set, a positive control set (only the inoculum of *V. harveyi* was added into the shrimp aquaria) and a treatment set by adding inoculate of a selected isolate and *V. harveyi* (biocontrol set). At 4-day intervals, the following parameters: water temperature, pH and salinity were directly measured in aquaria and the water samples were also collected to count viable cells of *V. harveyi*. *V. harveyi* was counted by the standard plate count with TCBS agar and incubated at 30°C for 24 h. Only green color colonies were counted as *V. harveyi*. Five shrimps were randomly measured their weight at the beginning and after 32 days of cultivation and the shrimp growth rate was calculated as described by Lobban *et al*. (1985) as equation: $G = (W_t/W_0) \times (100/t)$ where G: growth rate, W_t: body weight at time of sampling, W_0: body weight at the beginning of the cultivation period and t: time of sampling. Survival of the shrimp was also recorded during

cultivation. Multiple regression analysis and chi-Square test were used to analyze effects of antagonist inoculants against *V. harveyi* on shrimp growth rate and mortality. Means and standard deviations of triplicate are reported.

Results and discussion

1. Inhibition of *V. harveyi*

Only one of the strains, W3, of the bacterial isolates with strong proteolytic activity (W3, W6, W10, W30 and W310) produced an inhibitory effect on *V. harveyi*, with an inhibition zone of 21.62 mm (Table 1). This zone, produced by 70 µl of W3 culture broth, was similar to that produced by 25 µg sulphamethoxazole (20.02 mm) and 2 µg oxolinic acid (21.98 mm). However the inhibition zones produced by both tetracycline (30 µg) and norfloxacin (10 µg) were bigger, but this was expected as these antibiotics are commonly used to treat shrimp diseases (Holmström *et al*., 2003). Because an agar diffusion method was used to evaluate the antivibrio activity of the isolates and antibiotics it is possible that this may not be a true measure of their relative inhibitory effectiveness as the solubilities and diffusion characteristics of the antivibrio substances produced by the isolate W3 and the standard antibiotics might be very different. In addition the amounts of the W3 anti-vibrio substances and the antibiotics used could be very different. However, the results did show that the isolate W3, did inhibit the shrimp pathogenic bacterium, *V. harveyi*, and it was therefore interesting to investigate its possible use as a biological control agent during shrimp cultivation.

2. Bacterial identification and its characteristics

Strain W3, isolated from an intensive shrimp farm in Pattani province, was characterized according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). The results are presented in Table 2. This organism was a motile, Gram-negative, short rod (size 0.44 x 1.00 µm, based on SEM photographs). It was catalase, oxidase and citrate positive, but gave negative results for indole and the MR-VP test. This determined that W3 was a *Pseudomonas* sp. (Krieg and Holt, 1984; Holt *et al*., 1994). However, the conventional API 20E test results (Table 2) and also 16S rDNA analysis (data not shown) did not identify a species name for W3. However the sequences of the 16S rDNA showed 100% identity with strain *Pseudomonas* sp. AF 125317 that has the identity of *Pseudomonas* sp. AF 125317 pDL01 (no details of AF 125317 are available as the reference is to unpublished data) (www.ncbi.nlm.nih.gov/, 2005). Antibiotic sensitivity tests found that the isolate W3 was sensitive to most of the antibiotics used to control *Pseudomonas* spp., except for cephalothin and no haemolysis was detected (data not shown). The optimal growth temperature of W3 was between 28-30^{o}C (water temperature in shrimp ponds). This indicated that *Pseudomonas* sp. W3 could be part of the normal flora in a shrimp pond. Many Pseudomonads are normal flora in natural water and soil (Krieg and Holt, 1984) and are common inhabitants of marine environments (Vijayan *et al*., 2006). A similar result was found by Than *et al*. (2004) who found that 14 isolates of *Pseudomonas* spp, isolated from a marine environment produced protease and could

Table 1. Inhibition of *Vibrio harveyi* by antibiotics and the proteolytic bacterium W3

Proteolytic bacterium/antibiotic	Inhibition zone (mm)
W3 (70 µl)	21.62±0.40
Sulphamethoxazole trimethoprin (25 µg)	20.02±0.03
Tetracycline (30 µg)	26.05±0.80
Norfloxacin (10 µg)	24.97±0.21
Oxolinic acid (2 µg)	21.98±1.30

Values are mean ± standard deviation of six observations.

Table 2. Characteristics of the isolate W3 isolated from an intensive shrimp cultivation pond in Pattani province

Characteristic	Conventional Method	Test Kit (API 20 E)	(*Pseudomonas*)*
Shape	Short rod (0.44x1.00 μm)	ND	Rod
Gram stain	-	ND	-
Motility	+	ND	+
Catalase test	+	ND	+
Oxidase test	+	ND	D
Cirate test (CIT)	+	+	D
Indole test (IND)	-	-	ND
Methyl red test (MR)	-	ND	ND
Voges-Proskauer test (VP)	-	-	ND
H_2S production (H_2S)	-	-	ND
Urease (URE)	-	-	ND
Gelatin (GEL)	+	+	D
Oxidation-fermentation glucose	+/-	ND	ND
Oxidation-fermentation fructose	+ / -	ND	ND
Oxidation-fermentation lactose	- / -	ND	ND
D-glucose (GLU)	ND	+	D
D-mannitol (MAN)	ND	-	D
L-arabinose (ARA)	ND	-	D
Amygdalin (AMY)	ND	-	ND
D-sucrose (SAC)	ND	-	D
L-rhamnose (RHA)	ND	-	D
D-sorbitol (SOR)	ND	-	D
Inositol (INO)	ND	-	ND
Litmus milk	peptonization	ND	ND
L-arginine (ADH)	+	+	D
Nitrate reduction test	-	ND	D
L-ornithine (ODC)	-	-	D
L-lysine (LDC)	-	-	D
L-tryptophane (TDA)	ND	-	D
Water-soluble fluorescent pigment formed	+	ND	D
Marine Organisms	+	ND	D

*** = Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984); + = utilized, produced, motility; - = not utilized D = Different reactions occur in different taxa (species of a genus or genera of a family); ND = Not determined; Peptonization = Utilized protein**

inhibit the growth of *V. harveyi*. Moreover, many researchers have reported that *Pseudomonas* spp. are normal flora in shrimp ponds (Otta *et al*., 1999; Chythanya *et al*., 2002) and have been found in the gill, skin and liver of fish (Cahill, 1990).

3. Effect of the W3 isolate on *V. harveyi* during shrimp cultivation

For 5 of the 9 water sampling times there were no observed difference between the average numbers of *Vibrio* isolated from the biocontrol set and a positive control set. At the other 4 sampling times, although the observed average values were less in the biocontrol sample than the positive control, the only big difference was in the last sample at 32 days (Figure 1). Beginning with the same value of 5.5 log CFU *V. harveyi* /ml the pattern of behavior was almost identical over the

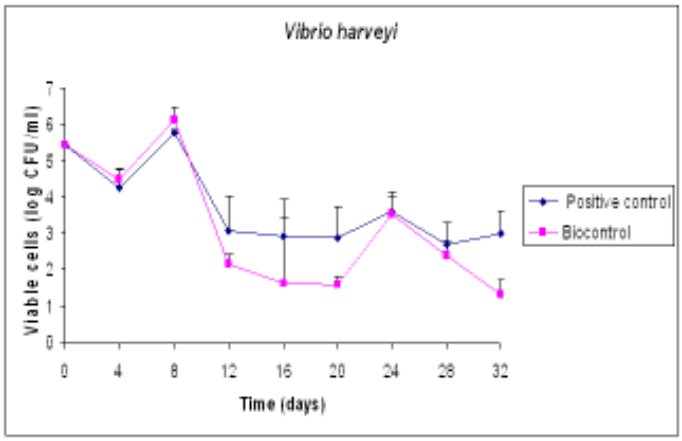

Figure 1. *V. harveyi* count in water samples from the positive control and the biocontrol aquaria during white shrimp cultivation

Table 3. Effect of isolate W3 inoculum to control *Vibrio harveyi* on the survival and growth rate of white shrimp in aquaria

Shrimp culture condition added with	Survival (%)	Growth rate for 32 days (%)
Vibrio harveyi	60±14.14 (ns)	9.28±0.13 (ns)
Isolate W3 + *V. harveyi*	67±7.07 (ns)	10.17±0.14 (ns)

Values are mean ± standard deviation of three observations.
ns = non significant difference

first 10 days. Then, at day 12 the numbers of *V. harveyi* decreased to an average of 2.1 and 3.0 log CFU/ml in the sets of the biocontrol and the positive control set respectively. This level of observed average difference of slightly more than 1 log was maintained over the 16 and 20 day samples, but these differences were not statistically significant ($p>0.05$). A 1.5 log difference at sampling time of 32 days was found. After day 2 there was a 40% shrimp mortality in the positive control set, whereas a 33% mortality was found in the biocontrol set at day 4 (Table 3). In contrast, no mortality of shrimp was found in the negative control set over 32 days of cultivation. From these results it seems that the isolate W3 could act as a biocontrol agent by delaying disease onset and reducing disease severity although no significant difference of survivor percentage of shrimp ($p > 0.05$) was found between the biocontrol set and the positive control set. Robertson *et al.* (1998) and Aguirre-Guzman *et al.* (2001) reported that infection of *P. vannamei* larvae with *V. harveyi* at 10^5 CFU/ml produced a larval disease called bolitas negricans or bolitas syndrome (a local name from Ecuador) (Vandenberghe *et al.*, 1999) and that could be one reason for the shrimp deaths in the present study.

Any infection of the shrimp by pathogenic *Vibrio harveyi* should result in an increase in the numbers of the pathogen in the culture medium. This may be the cause of the higher *Vibrio harveyi* numbers in the positive control, as shrimps in the biocontrol set appeared healthier and more active than those of the positive control set. It was unfortunate that the experiment was prematurely terminated by the failure of the power supply. Calculation of the shrimp growth rate at day 32 showed that in the biocontrol set (10.17%) was

higher than both control sets even though they were not statistically significant ($p>0.05$). The shrimp growth rate in the positive control set it was 9.28% and 9.44% in the negative control. This is another indication that the shrimp in the positive control set may have been more infected, causing growth retardation. A similar result was found by Sung *et al*. (1999) who reported that outbreaks of disease were associated with increases in the proportion of potentially pathogenic species in the *Vibrio* population of the culture pond water. Based on above results, no significant differences ($p>0.05$) for shrimp growth rate and numbers of *V. harveyi* were found between the biocontrol set and the positive control set and it can be explained that the same amount ($1x10^6$ CFU/ml) of *V. harveyi* and the isolate W3 was used in this study. Therefore, a higher inoculum of the isolate W3 to control *V. harveyi* will be investigated in a future work. The results indicate that treatment of shrimp cultures with the isolate W3 might be enhancing both growth and survival of shrimp.

Conclusion

A proteolytic bacterium, *Pseudomonas* sp. W3, has the potential for use as an inoculum to control *V. harveyi* during white shrimp cultivation. However, more work is required to devise the optimal conditions for producing antivibrio substances by this bacterium.

Acknowledgements

This work was supported by Graduate School, Prince of Songkhla University. The authors would like to thank Assoc. Prof. Dr.Kidchakan Supamattaya and Asst. Prof. Dr.Chutima Tantikitti, Aquatic Animal Health Research Center, Prince of Songkhla University for kindly supported aquarium and Mr.Surachai Ninrat for giving white shrimp. We also thank Dr.Putth Songsangjinda, Marine Shrimp Research and Development Center, and Dr.Brian Hodgson for their valuable suggestions.

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