



Screening and selection of bacteria inhibiting white spot syndrome virus infection to *Litopenaeus vannamei*



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ABSTRACT

A total of 173 bacterial strains were isolated from different sources at different regions such as fermented foods, shrimp guts, sea water, mangrove water, and sediments. These bacteria were screened against white spot syndrome virus (WSSV) infection in *Palaemon paucidens*. Based on mortality, white spot level, and healthiness, three bacterial strains were selected and identified using 16S rRNA gene sequencing. These bacterial strains were *Bacillus subtilis* KA1, *B. licheniformis* KA2, and *B. subtilis* KA3. WSSV challenge test in pilot scale was conducted using *Litopenaeus vannamei* with *B. subtilis* KA1 and *B. subtilis* KA3. The survival ratio of shrimp was 0% for WSSV control after 17th days, 84% for *B. subtilis* KA1 plus WSSV after 26th days, and 28% for *B. subtilis* KA3 with WSSV after 26th days. *B. subtilis* KA1 showed good growth at 18–37 °C in with and without 3% NaCl, and therefore can be applied to aquaculture at low to high temperatures. *B. subtilis* KA1 produced protease and lipase which can increase digestion to shrimp; exhibited antibacterial activity against *Vibrio parahaemolyticus*; and significantly increased the survival of WSSV challenged shrimps.

1. Introduction

Diseases are one of the major factors that increase shrimp mortality. Particularly, these diseases are caused by viruses, bacteria, fungi, parasites, and natural factor like algal blooms. Viral infections are the most crucial problem in aquaculture, wherein shrimps are mostly infected in their post-larval stages. In addition, certain viral infections cause mortality and deformations as well as obstruct growth. More than 20 viruses have been reported as pathogenic to shrimp, particularly white spot syndrome virus (WSSV). Considered as a serious pathogen, WSSV is a large DNA virus from Whispovirus and belongs to Nimaviridae family. WSSV is wide spread mostly shrimp producing countries, such as Bangladesh, Cambodia, China, India, Indonesia, and Korea, among others. In shrimp culture, WSSV promotes 80%–100% mortality within 8–10 days. It is present not only in shrimp, but also in all other crustaceans. WSSV is transmitted through cannibalism, death animals and contaminated water; similarly, birds transmit infected shrimps from one place to another [1]. The virus is known to occur in fresh, brackish, and marine water. WSSV multiplies due to environmental stress, temperature, pH, salinity, plankton blooming, molting, and spawning [2–4]. To prevent WSSV infection, several chemicals have been used particularly formaldehyde, malachite green, calcium oxide, and sodium hypochlorite [5]; but these chemicals pose

environmental hazards. Beneficial probiotic microbes can otherwise be used against WSSV infection they are a more environment friendly option, and can enhance survival and immunity condition of the shrimp.

Among the bacterial pathogens, *Vibrio* species cause vibriosis in penaeid shrimp [6]. Vibriosis -is caused by *Vibrio parahaemolyticus*, a Gram-negative halophilic, non-spore forming, curved rod-shaped bacterium that naturally lives in estuarine and marine environments worldwide [7]. Acute hepatopancreatic necrosis disease (AHPND) is a severe, newly emergent shrimp disease caused by *V. parahaemolyticus* that has already led to tremendous global losses in the shrimp culture industry [8,9].

Probiotics existing in microbial feed supplements can be beneficial to the host by improving its intestinal microbial balance [10]. With probiotic supplement treat, shrimp losses can be significantly reduced. Shrimp has a non-specific (innate) immune response; the vaccination (even if possible) can only provide short-term protection against pathogens, whereas probiotics provide extensive spectrum against disease. Shrimp culture mainly depends on the feed, and a bacterium producing protease and lipase is added into the culture pond. The enzymes increase the availability of nutrients during digestion, enhance growth rate and support survival. The use of low cost feed is also possible.

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In this study, we screened and selected bacterial strains. The strains inhibited WSSV infection to *Litopenaeus vannamei* and produced protease and lipase that increase feed digestion, and therefore can be applied as probiotics for shrimp aquaculture. The selected strain also exhibited anti-bacterial activity against *Vibrio parahaemolyticus*, thus causing significant survival of shrimp.

2. Materials and methods

2.1. Isolation of bacteria and culture

Various bacteria were isolated from different sources such as fermented food products (soybean paste and kimchi), shrimp guts, squid guts, mangrove water and sediments, and sea water using tryptic soy agar (TSA) containing 3% NaCl, pH 7.2 incubated at 37 °C for 2 days. A total of 173 morphologically different colonies were isolated. The isolated bacterium was grown in TSB containing 3% NaCl, 200 rpm, at 37 °C for 24 h; these cultures were used for inoculums (cell density 2×10^8) in WSSV challenge experiments.

2.2. Rearing of shrimp

Fresh water shrimp *Palaemon paucidens* and marine water shrimp *L. vannamei* were locally purchased and reared for screening and challenge tests. The experimental shrimps were tested for WSSV infections using 2-step PCR test [11], and the WSSV uninfected shrimp were used for the experiments. For the first screening, a glass tank (30 × 21.0 × 26.5 cm) contained 20 numbers of *P. paucidens* (0.2 g each) in 11 L underground water. The water was aerated for 2 days before the shrimps were added into the water and the shrimps adapted for 2–3 days and were maintained at 18 °C. For the second screening, 100 L culture tank containing 50 L sea-water and 30 numbers of *P. paucidens* (2 g each) was used. The shrimps adapted for 3 days and the animals were provided with proper aeration. Initially feeding was given with 7.5% of the body weight; then, subsequent feeding was adjusted to 5% of the body weight/day according to the leftover (unutilized feed). The feed was given thrice a day 40% at dawn (6.00 a.m.), 20% at dusk (2.00 p.m.), and 20% at night (10.00 p.m.). Throughout the experiment, the temperature of the tanks was maintained at 25 ± 1 °C using air conditioner. The bottom water in the tank along with excess feed and fecal was siphoned using 2 cm diameter sterilized plastic hose to enhance the survival of the shrimps.

2.3. Preparation of WSSV

One gram of WSSV-infected shrimp was homogenized in 9 mL of TNE buffer (Tris 50 mM, NaCl 100 mM and EDTA 1 mM, pH 7.4), the homogenized was centrifuged at 4000 rpm for 10 min and the supernatant was filtered with 0.25 µm syringe filter and the clear filtrate was stored at –70 °C for further study.

2.4. Screening of bacterial isolates against WSSV using *P. paucidens*

The 2 mL of bacterial culture broth was added into a glass culture tank containing 20 numbers of *P. paucidens* (0.2 g each), and 0.5 mL of WSSV extract was added into the culture tank. Based on screening, three bacteria (*B. subtilis* KA1, *B. licheniformis* KA2 and *B. subtilis* KA3) were selected and reconfirmed the WSSV effect in the culture tank containing 30 numbers of *P. paucidens* (2 g/shrimp). Experimental conditions were the following: tank 1 (-WSSV), tank 2 (+WSSV), tank 3 (*B. subtilis* KA1 + WSSV), tank 4 (*B. licheniformis* KA2 + WSSV) and tank 5 (*B. subtilis* KA3 + WSSV). The behavior of the shrimps was observed everyday as well as their survival ratio, level of white spot formation, overall health status, and the hardness of outer shell.

2.5. Evaluation of inhibition activity of selected isolates against WSSV in pilot scale using *L. vannamei*

Based on small-scale screening, 2 strains were tested for inhibition of WSSV infection in 50 L marine water with *L. vannamei*. The water was aerated for two days and 25 numbers of *L. vannamei* (6 g each) were reared in the 4 tanks (tank 1, *L. vannamei* control; tank 2, WSSV control; tank 3, *B. subtilis* KA1 + WSSV; and tank 4, *B. subtilis* KA3 + WSSV). The *L. vannamei* was adapted for 2–3 days; after that, 0.1% (cell density 2×10^8) of cultured bacterial isolate was inoculated into the culture tank. After 2 days, 0.01% of WSSV (10^4 copies) was directly added into the culture tank. During shrimp culture, shrimp mortality was monitored twice daily, and the dead shrimps were examined by PCR to confirm WSSV infection. The level of white spot formation, the overall health status, and hardness of outer shell were also observed.

2.6. Measurement of body weight and survival

$$\text{Average body weight (ABW)} = \frac{\text{Total weight}}{\text{Total number of animal}}$$

$$\text{Survival (\%)} = (\text{Final shrimp number} / \text{initial shrimp number}) \times 100$$

2.7. Detection of protease and lipase activity

The bacterial isolates were screened for protease and lipase activity. For protease, the agar plate contained peptone- 5, beef extract- 3, gelatin- 4, sodium chloride- 30, agar- 20 (g/L), pH 7.2. The bacteria inoculated plates were incubated overnight at 25 and 37 °C for 48 h. After incubation, the plates were stained with HgCl₂ and HCl solution (the 100 mL solution containing 15 g HgCl₂ and 20 mL HCl), and the protease producing strains were selected based on zone of clearance. The Rhodamine B mediums were used for lipase activity, the medium containing (%) beef extract- 0.5, peptone- 0.5, (NH₄)₂SO₄- 0.5, MgSO₄- 0.05, K₂HPO₄- 0.4, CaCl₂- 0.02, NaNO₃- 0.2, olive oil emulsion- 12, Rhodamine B (0.1 mg/mL)- 2, agar- 1.8, pH 6.5 and the plates were incubated at 25 and 37 °C for 48 h. The fluorescent hydrolyzed rings of orange color emitted by Rhodamine B around the colonies were observed under UV light.

2.8. Antimicrobial activity of the selected isolates against *Vibrio parahaemolyticus*

The antimicrobial activity of the selected isolates was assayed using agar diffusion test. Bacterial isolates were grown in TSB containing 3% NaCl at 37 °C in a rotary shaking incubator at 200 rpm for 24 h. *V. parahaemolyticus* was grown in nutrient broth containing 3% NaCl, 200 rpm at 37 °C for 24 h. The cells of *V. parahaemolyticus* (cell density 2×10^7) were spread on the agar plates. An 8 mm paper disc was put on top of the agar; then 50 µL culture supernatant of bacterial isolates (cell density 2×10^8) was dropped on the paper disk and incubated at 30 °C for 48 h. The diameter of inhibition zones around the colony was measured.

2.9. Identification of selected isolates

Three selected bacteria were identified by 16S rRNA gene sequencing using 27 F and 1492 R primer. Additionally the carbohydrate utilization of *B. subtilis* KA1 was examined using API 50 CHI kit (Biomérieux, Lyan, France) according to the manufacturer's instructions and the results were also used for identification.

Table 1

A: Survival and health condition of WSSV- infected fresh water shrimp *P. paucidens*, B: survival and health condition of WSSV- infected marine water shrimp *L. vannamei*.

A		Shrimp survival ratio (%) in days					Shrimp health conditions		
Conditions		3	6	9	12	15	White spot ^a	Overall health status ^b	Hardness of outer skin ^c
Control	-WSSV	100	86.7	66.7	60.0	60.0	-	±	++
	+ WSSV	100	73.3	53.3	20.0	0.0	++	±	±
Isolate + WSSV	<i>B. subtilis</i> KA1	100	86.7	66.7	56.7	46.7	±	++	++
	<i>B. licheniformis</i> KA2	100	76.7	60.7	45.0	26.7	+	+	++
	<i>B. subtilis</i> KA3	100	83.3	53.3	36.7	30.0	+	++	+

B		Shrimp survival ratio (%) in days						Shrimp health condition		
Conditions		6	16	20	21	24	26	White spot ^a	Overall health status ^b	Hardness of outer skin ^c
Control	-WSSV	100	96	8	8	8	8	-	+	++
	+ WSSV	96	96	0	0	0	0	++	±	±
Isolate + WSSV	<i>B. subtilis</i> KA1	100	96	84	84	84	84	±	++	++
	<i>B. subtilis</i> KA3	100	84	60	44	28	28	+	+	+

Note: ^a ++ many white spots, + medium, ± a few, - none. ^b ++ healthier and more actively moving, + healthy and actively moving, ± less healthy and less moving. ^c ++ hard, + medium, ± soft.

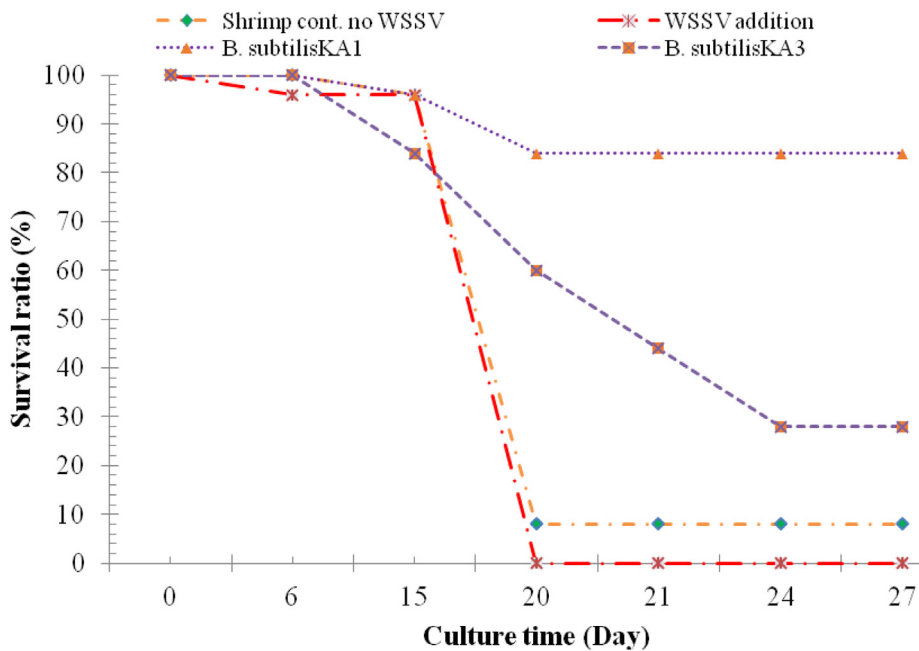


Fig. 1. Survival ratio of WSSV infected *L. vannamei*.

3. Results and discussion

3.1. Screening of bacteria efficiently inhibiting WSSV

The isolates were tested against WSSV infection with *P. paucidens*. Based on the survival ratio, white spot, healthiness, and hardness of skeleton, three bacterial isolates were selected. Survival ratio changed with time, and shrimp health conditions are shown in Table 1A. In shrimp control (without WSSV infection), the survival was 60% after 15 days. In WSSV control (without bacterial inoculums), all shrimp died (0% survival) after 15 days. In the tests of bacterial isolates with WSSV infection, the shrimp survival ratios were 26.7–46.7% based on the strains. The three isolates were identified by 16S rRNA gene sequencing, as *B. subtilis* KA1 (1491 bp), *B. licheniformis* KA2 (1515 bp), and *B. subtilis*KA3 (1492 bp). The *B. subtilis* KA1 was also subjected to a series of biochemical tests. With 97.1% identity, results confirmed the isolate

was *B. subtilis*.

3.2. Evaluation of selected bacterial isolates against WSSV in pilot scale using *L. vannamei*

Among the 3 selected isolates, 2 were selected based on survival ratio and shrimp health conditions. The selected *B. subtilis* KA1 and *B. subtilis* KA3 were tested further in marine water pilot scale experiment using *L. vannamei*. Results are shown in Table 1B and Fig. 1. The selection of bacteria improved not only the survival ratio, but also healthiness and hardness as well as reduced WSSV (Fig. 2). The white calcified spots appearing on the exoskeleton were easily identified, and these white spots are diagnostic of white spot disease [12].

In WSSV control, the mortality started on the 6th day, and cumulative mortality (0% survival) reached the 20th day. In the *B. subtilis* KA1 + WSSV, mortality was started on the 16th day and 84% survival

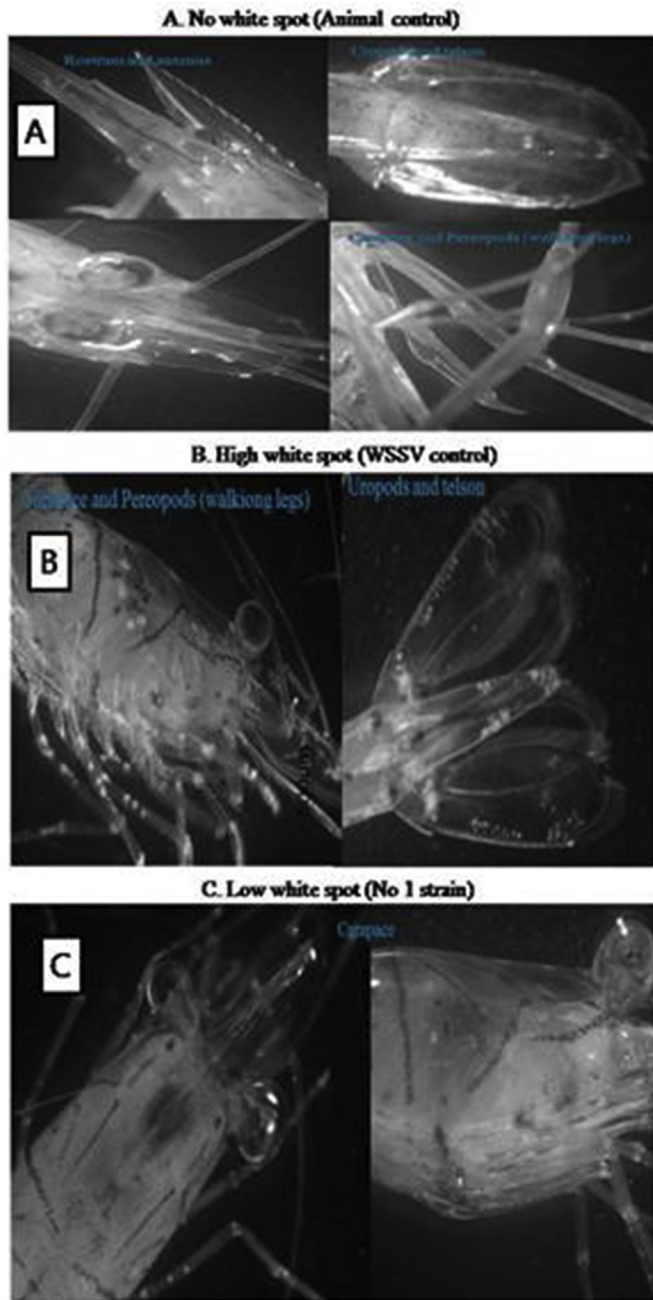


Fig. 2. WSSV infection: Animal control (A), WSSV control (B), and low infection with *Bacillus subtilis* KA1 (C).

was evident on the 26th day. In *B. subtilis*KA3 + WSSV, the mortality started on 16th days and 28% survival on 26th day of the experiment (Fig. 1). Hardness of skeleton was the same in control (-WSSV) and *B. subtilis* KA1 (+WSSV), whereas others were less hard. Healthiness was the best in *B. subtilis* KA1 (+WSSV), but less in others (Table 2B). At the start of the experiment, *L. vannamei* weighed 6.2 g/shrimp and it increase to after 15th days 7.5 g and 7.26 g in *B. subtilis* KA1 (+WSSV) and *B. subtilis* KA3 (+WSSV), respectively, and at 60th day increased 17 and 15 g, respectively. Rengpipat et al. [13] similarly reported that *Bacillus* S11 produced better yield, controlled the diseases, and improved the immunity in *Penaeus monodon*.

3.3. Cell growth and the activities of protease and lipase of the selected isolates

The three selected isolates grew well at 21–37 °C. At 18 °C, *B. subtilis* KA1 showed good growth, whereas *B. licheniformis* KA2 and *B. subtilis* KA3 grew poorly (Table 2). Since shrimp culture in Korea starts on May when water temperature is still low at 18 °C, the use of *B. subtilis* KA1 have more advantage than the two other strains. All the three isolates grew well in 3% NaCl and therefore can be used in marine water aquaculture.

All the three strains showed protease and lipase activities, which are both higher at 37 °C than at 25 °C. Among the isolates, *B. subtilis* KA1 produced the highest activities of protease and lipase. Shrimp feed contains 50–60% protease, 10% lipase, and others. These enzymes directly promoted growth and digestion and indirectly reduced (feed conversion ratio) feed taking. In addition, the main functions of the enzymes are increase of digestion (infection time the shrimp not taking feeds) and the reduction of mortality rate. Previous research [14] has reported protease producing *Bacillus* sp. Mk22 which enhanced shrimp survivals and reduced osmotic stress during *V. parahaemolyticus* and WSSV infection. *B. subtilis* produced high amount of secondary metabolites like antibiotics, fine chemicals and enzymes, as well as heterologous proteins, antigens and vaccines [15–17]. Microbial lipases are widely utilized because of their substrate specificity, availability, and catalytic activities [18].

3.4. Anti- vibrio activity of the selected isolates

B. subtilis KA1 showed relatively high activity (4 mm clear zone) against *V. parahaemolyticus*; whereas *B. licheniformis* KA2 and *B. subtilis* KA3 showed low activity (1 mm clear zone). *Vibrio* species are opportunistic pathogens and predominant in shrimp farming water under normal conditions [19]. Viruses are usually the main pathogens infecting shrimp as they reduce shrimp immunity, whereas superimposed bacterial infections accelerate shrimp mortality [20]. *V. parahaemolyticus* infection accelerated the proliferation of WSSV in *L. vannamei* and vice versa, and the combined proliferation of both *V. parahaemolyticus* and WSSV led to massive death of *L. vannamei* [21]. In addition to this superimposition, the opportunistic marine pathogen *V. parahaemolyticus* becomes highly virulent by acquiring a unique

Table 2 Cell growth at various temperatures in 3% NaCl, and protease and lipase activity.

Conditions	Cell growth at different temperature (°C)						Diameter of clear zone around colony (cm)			
	Cell grown without NaCl				Cell growth in 3% NaCl		Protease		Lipase	
	18	21	25	37	25	30	25	37	25	37
<i>B. subtilis</i> KA1	+	++	++	++	++	++	6	11	3	8
<i>B. licheniformis</i> KA2	±	++	++	++	++	++	5	6	< 1	7
<i>B. subtilis</i> KA3	±	++	++	++	++	++	4	5	< 1	5

AHPND-associated plasmid that expresses a deadly toxin [22]. Probiotics and competitive exclusion agents are considered to enhance gut microflora by preventing the colonization of the gastrointestinal tract by pathogenic bacteria [23,24].

In conclusion, *B. subtilis* KA1 is able to control both WSSV and *V. parahaemolyticus*. *B. subtilis* KA1 also showed good growth at 18–37 °C and in 3% NaCl. Therefore, it can be applied to marine aquaculture at low to high temperatures. *B. subtilis* KA1 produced higher levels of protease and lipase which can increase feed digestion. Hence, *B. subtilis* KA1 obtained from this investigation shows strong potential for application as probiotics to shrimp aquaculture.

Conflicts of interest

There is no conflict of interest.

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