Curr Microbiol (2007) 55:409–412
DOI 10.1007/s00284-007-9000-0

Inhibitory Activity of Probiotic *Bacillus subtilis* UTM 126 Against *Vibrio* Species Confers Protection Against Vibriosis in Juvenile Shrimp (*Litopenaeus vannamei*)

José Luis Balcázar · Tyrone Rojas-Luna

Received: 6 March 2007 / Accepted: 20 May 2007 / Published online: 7 August 2007

Abstract The bacterial strain *Bacillus subtilis* UTM 126 produced antimicrobial activity against pathogenic *Vibrio* species, including *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi*. The probiotic effect of *B. subtilis* was tested by feeding juvenile shrimp (*Litopenaeus vannamei*) food supplemented with *B. subtilis* (10^5 CFU/g) for 28 days before an immersion challenge with *V. harveyi* at 10^5 CFU/mL for 24 h. The treatment with *B. subtilis* UTM 126 decreased final mortality to 18.25%, compared with 51.75% in the control group. *Bacillus subtilis* UTM 126 has potential applications for controlling pathogenic *V. harveyi* in shrimp aquaculture.

Keywords Pathogenic control · Probiotic · Shrimp · Vibriosis

Vibrio species are among the most important bacterial pathogens of cultured shrimp. They are responsible for several diseases, and mortalities of up to 100% due to vibriosis have been reported [8]. Using antibiotics in aquaculture for the prophylactic treatment of diseases has potential negative consequences, particularly drug resistance arising in microorganisms through adaptation or by genetic exchange [9]. In the search for more effective and environmentally friendly treatments, probiotics have emerged as a viable alternative [1, 18].

J. L. Balcázar (✉)
Laboratory of Fish Pathology, Faculty of Veterinary Sciences, University of Zaragoza, Zaragoza, Spain
e-mail: balcazar@unizar.es

T. Rojas-Luna
Faculty of Aquaculture, Technical University of Machala, Machala, Ecuador

Probiotics are defined as live microorganisms that confer a health benefit on the host when consumed in adequate amounts [12]. In order to be considered as biological control agents in aquaculture, probiotics should be non-pathogenic and biochemically and physiologically well characterized. They should be normal inhabitants of the host and able to survive and grow at the site of application while exerting their beneficial effect. Finally, they should maintain their viability and activity throughout product manufacturing and storage [2, 7].

The genus *Bacillus* has been isolated from crustacean intestine [13], bivalves [15], and marine fish [16]. Some species of this genus have shown inhibitory activity against various pathogens [14, 16]. The present study aimed specifically to investigate the probiotic effects of *Bacillus subtilis* UTM 126 on *Vibrio harveyi* in juvenile shrimp (*Litopenaeus vannamei*).

Materials and Methods

Bacterial Strains

Bacillus subtilis strain UTM 126, isolated from shrimp culture ponds by dilution plating on marine agar (Difco, Detroit, MI, USA), was identified by using standard morphological and physiological techniques and the API 50CH system (bioMérieux, Marcy-l'Etoile, France).

A virulent *Vibrio* strain, *V. harveyi* TR51, which was isolated in Ecuador in 2002 from *L. vannamei* larvae showing clinical symptoms of vibriosis, was used in the infectivity experiments. In addition, three strains isolated from diseased shrimp (*V. alginolyticus* UTM 102, *V. harveyi* EC11, and *V. parahaemolyticus* PS-017) and two reference strains (*V. harveyi* ATCC 14126 and ATCC

33843) were used as indicator bacteria in the antimicrobial activity assays. All strains were stored in Luria-Bertani broth (LB; Difco) with sterile glycerol (15% v/v).

Antimicrobial Activity Assay

Cultures of *B. subtilis* UTM 126 grown in 100 mL LB broth for 24 h at 28°C was centrifuged at 10,000*g* for 10 min. The supernatant was sterilized by passage through a 0.45-μm Millipore filter (Millipore, Bedford, MA, USA), and neutralized (pH 6.8) with 5 N NaOH. The indicator strains were subcultured on trypticase soy agar (TSA; Difco) for 12 h at 28°C. Plates of Mueller-Hinton agar (MH; Difco) were flooded with 100 μL of indicator bacteria and air-dried for 15 min before disks impregnated with 10 μL of filtered supernatant were positioned on them. Disks impregnated with LB broth (pH 5.6) and neutralized LB broth were used as controls to determine possible inhibitory activity of the medium. The diameter of the clear zone around each disk was measured after controlled incubation for 24 h at 28°C. All experiments were carried out in triplicate to ensure feasibility and reproducibility.

Preparation of the Feed

Bacillus subtilis UTM 126 was grown in LB broth in a shaking incubator at 28°C overnight. After incubation, the cells were harvested by centrifugation (2000*g*), washed twice with phosphate-buffered saline [PBS; 10 mM sodium phosphate, 150 mM sodium chloride (pH 7.2)], and resuspended in the same buffer. The absorbance at 600 nm was adjusted to 0.25 ± 0.05 in order to standardize the number of bacteria (10^7–10^8 CFU/mL). Dilution plating was used to verify the relationship between absorbance at 600 nm and CFU per milliliter.

Commercial shrimp feed (Balanfarina SA, Ecuador) was used as the basal diet for the supplementation of *B. subtilis* UTM 126. In order to reach a final concentration of 10^5 CFU/g feed, bacterial suspensions were slowly applied into the feed, mixing part by part in a drum mixer. The amount of *B. subtilis* UTM 126 in the feed was determined by plate counting on TSA agar.

Probiotic Treatment and Infection of Shrimp

Shrimp (*L. vannamei*) were obtained from a commercial shrimp hatchery in the Province of El Oro, Ecuador. The shrimp were maintained in a water bath thermostatically controlled at 28°C. The shrimp had not been exposed to shrimp diseases and were deemed pathogen-free by standard microbiological techniques. Shrimp were acclimatised for 5 days before use in order to ensure adequate health.

After the acclimation period, the average weight of the shrimp was 1.20 g and the shrimp were divided into eight 200-L tanks, each containing 50 shrimp. Four tanks were treated with feed supplemented with 10^5 CFU/g of *B. subtilis* UTM 126 for 28 days; the other four tanks served as the control group and were fed with a regular diet during the entire trial period. Shrimp in all groups were fed twice daily at 3.0% of biomass.

The water temperature was held at 28 ± 1°C during the whole trial. The weight and the general health of the shrimp were recorded and 10 shrimp were removed for microbiological examination at the end of 28 days.

After 28 days of probiotic supplementation, the experimental infection was carried out by the immersion method. *V. harveyi* TR51 was grown for 12 h at 28°C in LB broth. Shrimp in all eight of the tanks were exposed to *V. harveyi* TR51 (10^5 CFU/mL) for 24 h. After infection, the shrimp were kept under the initial experimental conditions. The accumulated mortality of the shrimp was recorded. At the end of the study, *B. subtilis* UTM 126 from the hepatopancreas were enumerated by growth on MYP agar (Difco), a selective medium for the isolation of the *Bacillus* species.

Statistical Analysis

The results were analyzed by the Student's *t*-test to determine differences ($P < 0.05$) between tested groups. All statistics were performed with SPSS for Windows, version 11.5 (SPSS Inc, Chicago, IL, USA).

Results

Antimicrobial Activity Assay

The filtered supernatant of *B. subtilis* UTM 126 exhibited greater inhibitory activity against three of the four strains of *V. harveyi* than it did against *V. alginolyticus* or *V. parahaemolyticus* isolated from diseased shrimp (Table 1). The inhibitory zones around the test disks with *B. subtilis* supernatant were about 10–15 mm in diameter.

Probiotic Treatment and Infection of Shrimp

We did not observe mortalities during 28 days of probiotic supplementation. Moreover, no significant difference ($P = 0.68$) was observed in the mean final weight between the different groups. The mean final weight was 2.56 g in the groups supplemented with *B. subtilis* UTM 126 and 2.52 g in the control groups.

To investigate whether *B. subtilis* UTM 126 is able to protect shrimp against vibriosis infection, shrimp were infected with *V. harveyi* by the immersion method. The final

Table 1 Antimicrobial activities of *B. subtilis* UTM 126 towards indicator bacteria

Strain	Activity[a]
V. alginolyticus UTM 102	+
V. harveyi TR51	+
V. harveyi EC11	++
V. harveyi ATCC 14126[b]	++
V. harveyi ATCC 33843[b]	++
V. parahaemolyticus PS-017	+

[a] Clear zones: +, clear zone of 10–15 mm; ++, clear zone of 15 mm or more

[b] Type strain

Sources: ATCC, American Type Culture Collection; UTM 102, TR51, EC11 and PS-017, collection of the Laboratory of Aquaculture, Technical University of Machala, Ecuador

mortality of shrimp treated with *B. subtilis* UTM 126 was 18.25%; whereas mortality was 51.75% in shrimp not treated with probiotics (Table 2). Statistical analysis demonstrated significant differences ($P < 0.001$) in mortality between *B. subtilis* UTM 126 and control groups. Mortality was observed in both *B. subtilis* UTM 126 treatments and controls starting on the second day of contact with the pathogen, but all *B. subtilis* UTM 126 treatments were significantly different from the control when the mortality stabilized on the 11th day. At the end of the study, bacteriological analysis found $2.7 \times 10^5 \pm 4.1 \times 10^4$ CFU/g *B. subtilis* UTM 126 in the hepatopancreas of treated shrimp, whereas control groups showed < 42 CFU/g *Bacillus* species.

Discussion

In this study, we determined that *B. subtilis* UTM 126 inhibited growth of *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi*. The inhibitory mechanism of the interaction was not characterized in this study. However, previous studies have suggested that the inhibitory effects of *Bacillus* might be due to either alteration of pH in the growth medium, utilization of essential nutrients, or production of volatile compounds [3, 6, 19]. In addition, several studies have reported that *Bacillus* produces polypeptide antibiotics, such as bacitracin, gramicidin S, polymyxin, and tyrotricidin, which are active against a wide range of Gram-positive and Gram-negative bacteria [4, 10, 11].

Manipulation of endogenous microbiota via the administration of probiotics might constitute a valuable mechanism to increase shrimp growth and survival rates. Although there were no significant differences in the final weight between groups, possibly due to the short period of the study, we observed that probiotic supplementation was

Table 2 Mortality of *L. vannamei* juveniles following challenge by immersion with *V. harveyi* after feeding with and without *B. subtilis* UTM 126 for 2 weeks

Treatment	Mortality (%)[a]
B. subtilis UTM 126	18.25 ± 2.39*
Control	51.75 ± 4.25

[a] Values are means with standard errors of four replicates for each treatment (mean ± SE; $n = 4$)

*$P < 0.001$ as determined by Student's *t*-test (*B. subtilis* UTM 126 vs. control)

effective in reducing shrimp mortality from *V. harveyi* infection. It is therefore reasonable to speculate that action mechanism of *B. subtilis* UTM 126 is based on competitive exclusion of the pathogen, because the microbiological analysis revealed the presence of *B. subtilis* in the shrimp hepatopancreas at the end the study. Gomez-Gil et al. [5] demonstrated a wide diversity of microorganisms, particularly *Vibrio* species, living in the hepatopancreas of healthy juvenile *L. vannamei*. Thus, the composition of hepatopancreas microbial communities constitutes a crucial aspect of health maintenance and disease prevention.

Vaseeharan and Ramasamy [17] found that growth of pathogenic *V. harveyi* in tiger shrimp was controlled by the probiotic effect of *B. subtilis* BT23 *in vitro* and *in vivo*. Disease resistance was improved and accumulated mortality was reduced by 90% when juvenile *Penaeus monodon* were exposed to *B. subtilis* BT23 isolated from shrimp culture ponds before a challenge with *V. harveyi.* Similarly, Rengpipat et al. [14] reported that inoculation with *Bacillus* S11, which had previously demonstrated its inhibitory effect *in vitro* against *V. parahaemolyticus* and *V. harveyi*, resulted in greater survival of *P. monodon* challenged with pathogenic luminescent bacteria.

The ability of *B. subtilis* UTM 126 to suppress pathogen growth *in vitro* and *in vivo* conditions suggests that it is a promising probiotic candidate. Future challenge experiments in shrimp could provide valuable insight into its potential probiotic effect in situations directly relevant to aquaculture conditions.

Acknowledgments This study was supported by the Ecuadorian government through the IECE.We thank Dr. Alison Moldenke for helpful and critical discussions.

References

1. Balcázar JL, de Blas I, Ruiz-Zarzuela I, Cunningham D, Vendrell D, Múzquiz JL (2006) The role of probiotics in aquaculture. Vet Microbiol 114:173–186
2. Balcázar JL, Decamp O, Vendrell D, de Blas I, Ruiz-Zarzuela I (2006) Health and nutritional properties of probiotics in fish and shellfish. Microb Ecol Health Dis 18:65–70

3. Chaurasia B, Pandey A, Palni LMS, Trivedi P, Kumar B, Colvin N (2005) Diffusible and volatile compounds produced by an antagonistic *Bacillus subtilis* strain cause structural deformations in pathogenic fungi in vitro. Microbiol Res 160:75–81
4. Drablos F, Nicholson D, Ronning M (1999) EXAFS study of zinc coordination in Bacitracin A. Biochim Biophys Acta 1431:433–442
5. Gomez-Gil B, Tron-Mayén L, Roque A, Turnbull JF, Inglis V, Guerra-Flores AL (1998) Species of *Vibrio* isolated from hepatopancreas, haemolymph and digestive tract of a population of healthy juvenile *Penaeus vannamei*. Aquaculture 163:1–9
6. Gullian M, Thompson F, Rodríguez J (2004) Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. Aquaculture 233:1–14
7. Hansen GH, Olafsen JA (1999) Bacterial interactions in early life stages of marine cold water fish. Microb Ecol 38:1–26
8. Karunasagar I, Pai R, Malathi GR, Karunasagar I (1994) Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture 128:203–209
9. Moriarty D (1997) The role of microorganisms in aquaculture ponds. Aquaculture 151:333–349
10. Morikawa M, Ito M, Imanaka T (1992) Isolation of a new surfactin producer *Bacillus pumilus* A-1, and cloning and nucleotide sequence of the regulator gene, psf-1. J Ferment Bioeng 74:255–261
11. Perez C, Suarez C, Castro GR (1993) Antimicrobial activity determined in strains of *Bacillus circulans* cluster. Folia Microbiol 38:25–28
12. Reid G, Sanders ME, Gaskins HR, Gibson GR, Mercenier A, Rastall RA, Roberfroid MB, Rowland I, Cherbut C, Klaenhammer TR (2003) New scientific paradigms for probiotics and prebiotics. J Clin Gastroenterol 37:105–118
13. Rengpipat S, Rukpratanporn S, Piyatiratitivorakul S, Menasaveta P (2000) Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture 191:271–288
14. Rengpipat S, Phianphak W, Piyatiratitivorakul S, Menasaveta P (1998) Effects of a probiotic bacterium in black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture 167:301–313
15. Sugita H, Tanaami H, Kobashi T, Deguchi Y (1981) Bacterial flora of coastal bivalves. Bull Jpn Soc Sci Fish 47:655–661
16. Sugita H, Hirose Y, Matsuo N, Deguchi Y (1998) Production of the antibacterial substance by *Bacillus* sp. strain NM12, an intestinal bacterium of Japanese coastal fish. Aquaculture 165:269–280
17. Vaseeharan B, Ramasamy P (2003) Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett Appl Microbiol 36:83–87
18. Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 64:655–671
19. Yilmaz M, Soran H, Beyatli Y (2006) Antimicrobial activities of some *Bacillus* spp. strains isolated from the soil. Microbiol Res 161:127–131