

Isolation and characterisation of *Bacillus* spp. antagonistic to *Vibrio parahaemolyticus* for use as probiotics in aquaculture

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Abstract Acute gastroenteritis caused by pathogenic *Vibrio parahaemolyticus* is one of the major factors affecting the development of aquaculture and the safety of seafood. Using the antagonism of probiotics against pathogens is an alternative strategy to antibiotics and a common trend to control food-borne pathogenic bacteria. In this study, a total of 249 isolates were isolated from four types of seafood (*Litopenaeus vannamei*, *Oratosquilla oratoria*, *Macrura veneriformis* and *Portunus trituberculatus*) and coastal sediment from Liaodong Bay in the Bohai Sea, China with five different separation agars. The most isolates came from the sample of coastal sediment and on agar of 2216E, which accounted for 36.14 and 54.62 % respectively. Twenty-four among 249 isolates displayed direct antimicrobial activity to *V. parahaemolyticus* with spot inoculation. Sixteen active isolates were selected for extracellular antimicrobial activity using the Oxford cup

method. Only strains of B16 and J7 showed extracellular antimicrobial activity and were identified as *Bacillus pumilus* and *Bacillus mojavensis* respectively based on the physiological identification and 16S rRNA sequence analysis. Both of the strains B16 and J7 exhibited extracellular hydrolytic enzyme activity and antagonism against more than one indicator bacteria in vitro, which indicates that the two strains have broad potential application as suitable probiotic candidates in aquaculture while *B. mojavensis* was first reported to inhibit pathogenic *Vibrio* spp. in vitro. There is no particular trait as to antagonism of *B. pumilus* B16 or *B. mojavensis* J7 to Gram-positive or Gram-negative indicator bacteria.

Keywords Antagonism · Probiotics · *Bacillus* spp. · *Vibrio parahaemolyticus*

Introduction

With the increase in seafood consumption around the world, the occurrence of seafood safety events presents a rising trend in recent years (Fernandez-Piquer et al. 2011; Xu et al. 2012). Acute gastroenteritis caused by pathogenic *Vibrio* spp. is one of the major factors limiting the development of the marine economy. Among them, *Vibrio parahaemolyticus* has become a primary causative factor of food-borne disease outbreaks in coastal countries worldwide (Zarei et al. 2012), including China, Japan, India, Thailand, Australia, and the United States (Wu et al. 2014; Raghunath et al. 2008; Iwamoto et al. 2010; WHO/FAO 2011; Yu et al. 2013): this affects the safety of seafood and the health of consumers. *Vibrio parahaemolyticus* is a natural bacterium in marine and estuarine environments, the animals carrying *V. parahaemolyticus* could

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be the principal vehicle transmitting the pathogenic bacterium to humans, including aquatic farmed animals (Griffitt et al. 2011).

Currently, bacterial diseases in aquatic farmed animals are generally controlled by antibiotics. The long-term use of antibiotics can cause environment pollution and threaten human health. Using probiotics antagonistic to *V. parahaemolyticus* is an alternative strategy to antibiotics and a common trend to control the food-borne pathogen *V. parahaemolyticus* (Touraki et al. 2012; Aranda et al. 2012). The concept of probiotics has been constantly enriched from "...a live microbial feed supplement..." (Fuller 1989) to "...any microbial probiotics or the product of the microorganisms (not necessarily live) that are beneficial to the health of the host..." (Salminen et al. 1999). The effect of probiotics on aquatic pathogenic bacteria was mainly manifested in the following ways: competitive exclusion (binding sites, nutrition, energy, and iron carrier), digestion enhancement in its host, production of antimicrobial active substances (such as friendly antibiotics, bacteriolytic enzymes, and bacteriocins), strengthening of aquaculture animal non-specific innate immunity, water quality improvement, etc. (Newaj-Fyzul et al. 2014).

Bacillus species is the oldest type of bacterium known and is widely distributed in the natural world, including marine environments (Nithya et al. 2010). This genus of bacteria has been differentiated in more detail by new classification methods such as 16S rRNA sequence analysis. Many species within this genus have been proven to be safe, even as fermentation strains of food and as probiotics drugs for oral consumption (Bacon and Hinton 2002). Some reports stated that *Bacillus* could produce biological control agents and has become one of the most important sources of probiotics (Ravi et al. 2007; Barman et al. 2011; Zokaefar et al. 2012; Donio et al. 2014). However, the application of *Bacillus* spp. as a probiotic in aquaculture remains limited.

To develop *Bacillus* spp. into a probiotic to control pathogenic *V. parahaemolyticus* in aquaculture, strains were isolated and screened from seafood and the coastal sediment from Liaodong Bay of Bohai Sea, China. The active isolates were identified and investigated for hydrolytic exoenzymatic activity and their antagonistic spectra respectively. The bacteriostatic activity of the culture and the supernatant of active isolates were surveyed to uncover novel antibacterial ingredients.

Materials and methods

Sample collection

Five different coastal sediment samples were collected from the exposed intertidal zone of Liaodong Bay, in the

north of the Bohai Sea, Jinzhou, China, using a shovel, at a temperature of around 3 °C in March, 2013. Each cuboidal sediment sample measured approximately 8 cm (side length) and they were taken from different sampling sites including: 40°49'29.64"N, 121°03'53.10"E, 40°49'41.07"N, 121°04'26.61"E, 40°49'13.67"N, 121°04'15.62"E, 40°48'53.56"N, 121°04'25.59"E, and 40°48'40.34"N, 121°04' 36.87"E. Seafood samples (5 kg each) of *Litopenaeus vannamei*, *Oratosquilla oratoria*, *Macrura veneriformis*, and *Portunus trituberculatus* were obtained from a local aquatic products wholesale market in Jinzhou. The four types of seafood were selected because they were among the favourite local foods and were easily obtained. Each separate sample was placed in a sterile plastic bag without seawater and transported to the laboratory within 2 h and processed immediately to isolate the relevant microorganisms.

Isolation of bacteria

The coastal sediment and the intestines of four types of seafood and the gills of two types (*O. oratoria* and *P. trituberculatus*) were selected as experimental samples. Five different sediment samples were individually removed from the outer layer with a sterile scalpel and then mixed evenly. Four kinds of seafood were aseptically shucked to collect the intestines or the gills separately. Some 25 g of each sample (wet mass) were homogenised in 225 ml 0.85 % sterile saline solution following tenfold serial dilution to 10⁴ times. Some 100 µl of each dilution was spread on five specialised media plates respectively, and in triplicate, to get as many bacteria as possible. The five types of separation medium respectively were as follows: (1) all purpose Tween (APT) agar (Qingdao Hope Bio-Technology Co. Ltd, Qingdao, China), (2) thiosulphate citrate bile saccharose (TCBS) agar (Qingdao Hope Bio-Technology Co. Ltd, Qingdao, China), (3) nalidixic acid cetrinide (NAC) agar (Beijing Land Bridge Technology Co. Ltd, Beijing, China), (4) marine agar 2216E (2216E, prepared by the authors), and (5) manganese nutrient agar (Mn²⁺-NA, prepared by the authors). Their compositions are listed in Table 1. All of the plates were incubated at 28 °C for 24–48 h until the morphology of the colony could be distinguished.

Direct antimicrobial activity assay

The antimicrobial activity of all isolates against *V. parahaemolyticus* was assayed with spot inoculation. The *V. parahaemolyticus*, with its thermo-stable direct hemolysin related hemolysin (tdh⁻/trh⁺) gene (ATCC 17802), was incubated in alkaline peptone water (APW, Qingdao Hope Bio-Technology Co. Ltd, Qingdao, China) and shaken at

Table 1 Compositions and concentrations of five types of separation medium used in this study

Media	Compositions ^a (g L ⁻¹)
APT	Yeast extract 7.5 g, Pancreatic digest of casein 12.5 g, Glucose 10 g, Sodium citrate 5 g, NaCl 5 g, Thiamine-HCl 0.01 g, K ₂ HPO ₄ 5 g, MnCl ₂ 0.14 g, MgSO ₄ 0.8 g, Fe ₂ (SO ₃) ₃ 0.02 g, FeSO ₄ 0.04 g, Tween-80 0.2 g, pH 6.7
TCBS	Yeast extract 5 g, Peptone 10 g, Na ₂ S ₂ O ₃ 10 g, Sodium citrate 10 g, Ox gallbladder powder 5 g, Sodium taurocholate 3 g, Sucrose 20 g, NaCl 10 g, Ferric citrate 1 g, Bromothymol blue 0.04 g, Thymol blue 0.04 g, pH 8.6
NAC	Peptone 20 g, MgSO ₄ 0.2 g, CTAB (cetyl trimethyl ammonium bromide) 0.2 g, K ₂ HPO ₄ 0.3 g, Nalidixic acid 0.015 g, pH 7.4
2216E	Peptone 5 g, Yeast extract 1 g, Ferric citrate 0.1 g, pH 7.6
Mn ²⁺ -NA	Peptone 10 g, Beef extract 3 g, NaCl 5 g, 3.08 % Manganese sulfate solution 1 ml, pH 7.3

^a All the media contained 15 g L⁻¹ agar and 1000 ml distilled water except for 2216E which contained 1000 ml aged seawater

180 rpm and 28 °C overnight and diluted to 10⁵ CFU/ml with 0.85 % sterile saline solution for use as an indicator in the direct antimicrobial activity assay. All isolated strains were spot inoculated (Chahad et al. 2012) in triplicate on nutrient agar (Beijing Land Bridge Technology Co. Ltd, Beijing, China) plates coated with *V. parahaemolyticus* in advance and incubated at 28 °C for 24–48 h. The inhibitory zone diameters were measured with a Vernier caliper.

Extracellular antimicrobial activity assay

The antimicrobial activity of cultures and supernatants from the isolates were determined by the Oxford cup method. In detail, the isolates antagonistic to *V. parahaemolyticus* in the direct antimicrobial activity assay were respectively inoculated to a 250 ml conical flask filled with 100 ml of nutrient broth (Beijing Land Bridge Technology Co. Ltd, China) and incubated at 28 °C, while being shaken at 180 rpm for 24 h. A part of the culture was collected as one of the samples. Another part of the culture was centrifuged at 8,000 rpm (Thermo Scientific Sorval Biofuge Stratos 75005289, Germany) at 4 °C for 10 min. The supernatant was sterilised through 0.22 µm pore-size filters as for the other sample. Some 180 µl of the culture and the supernatant were added into the Oxford cup in the nutrient agar inoculated with 10⁵ CFU/ml of indicator bacterium *V. parahaemolyticus* in triplicate respectively and the same amount of liquid medium was used as a blank control. All the plates were incubated at 28 °C for 24 h and the size of the inhibiting zones around the cup was measured thereafter. Those strains exhibiting extracellular antimicrobial activity were stored at -80 °C with 15 % glycerol as potential probiotics.

Morphological and physiological characteristics of potential probiotic strains

The potential probiotic strains were studied to determine their morphological (Gram staining), cultural (colony), and

physiological (biochemical test) characteristics. Physiological identification was undertaken as described by Bergey's Manual of Systematic Bacteriology using a trace biochemical identification reaction tube (Hangzhou BinHe Microorganism Reagent Co. Ltd, Hangzhou, China). The results of biochemical tests were interpreted according to the identification code book (Hangzhou BinHe Microorganism Reagent Co. Ltd, Hangzhou, China).

16S rRNA gene amplification, sequencing, and analysis of potential probiotic strains

The DNA template of potential probiotic strains was obtained using a bacterial genome DNA rapid extraction kit (Sangon Biotech Co. Ltd, Shanghai, China). Polymerase Chain Reaction was carried out using the 16S universal primers: 16S-27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-1492R (5'-TACGGCTAC CTTGTTACGACTT-3'). The primers were synthesised by Sangon Biotech Co. Ltd, Shanghai, China. PCR was performed in a 50 µl reaction system consisting of 25 µl 2 × Taq PCR Master Mix (Sangon Biotech Co. Ltd, Shanghai, China), 1 µl DNA template, 2 µl Primer F, 2 µl Primer R, and 20 µl sterile ddH₂O. The PCR was performed under the conditions pertaining to: initial denaturation at 94 °C for 2 min, denaturation at 94 °C for 1 min, renaturation at 60 °C for 1 min, extension at 72 °C for 95 s with a total of 30 cycles, and the final extension at 72 °C for 10 min. The purity of PCR products was observed by 1 % agarose gel electrophoresis. The sequencing of the 16S rRNA gene was performed by Sangon Biotech Co. Ltd, Shanghai, China, with 27F and 1492R primers. The RNA sequences were analysed through <http://www.ncbi.nlm.nih.gov/> blast by comparing them with bacterial 16S rRNA sequences in Genbank to determine the genus to which each isolate belonged. The 16S rRNA sequences of standard strains within this genus were downloaded and aligned with those of potential probiotic strains using MEGA 5.05 software. The phylogenetic tree was constructed with a neighbour-

joining DNA distance algorithm to determine the evolutionary distance between strains.

Enzymatic assay

The hydrolytic exoenzymatic activities of potential probiotics were further detected by using the agar diffusion method. Nutrient agar, as the experimental medium, was added to different substrates according to Nair et al. (2012) with minor modifications, including skimmed milk for proteolytic activity, soluble starch for amylolytic activity, and 1 % Tween 80 for lipolytic activity, respectively. The hydrolytic exoenzymatic activity of the strains inoculated on the plates were observed after incubating at 28 °C for 24 h. Proteolytic activity and lipolytic activity were judged by the transparent circle of milk degradation and the halo formation of precipitated fatty acids around the colony. However, amylolytic activity was tested with 1 % iodine solution.

Antagonistic spectrum of potential probiotic strains

The antagonistic spectrum of the culture and the supernatant of potential probiotics were investigated using other aquaculture or human pathogens, including *V. parahaemolyticus* with the thermostable direct hemolysin (tdh^+/trh^-) gene (ATCC 33847), *Vibrio vulnificus* (environmentally isolated), *Vibrio harveyi* (environmentally isolated), *Salmonella typhimurium* (CMCC 50115), *Pseudomonas aeruginosa* (ATCC 9027), *Aeromonas hydrophila* (environmentally isolated), *Listeria monocytogenes* (ATCC 19115), *Bacillus subtilis* (CMCC 63530), *Escherichia coli* (CMCC 44102) and *Staphylococcus aureus* (CMCC 26003). These additional assays were performed with Oxford cup method by using nutrient agar medium and incubated at 28 °C for 24 h. Considering that the majority of isolates were from the natural environment, we selected environmental microbial cultivation temperatures. The pathogens mentioned above were incubated separately in the nutrient broth and shaken at 150 rpm and 37 °C overnight except for *Vibrio* spp. for which a temperature of 28 °C was used.

Results and discussion

Effect of samples and separation media on the recoverability of bacteria

A total of 249 bacteria were isolated from five samples of coastal sediment and the intestines or gills of *L. vannamei*, *O. oratoria*, *M. veneriformis*, and *P. trituberculatus* by using five types of separation media of APT, TCBS, NAC, 2216E, and Mn^{2+} -NA respectively. According to the

sample source and separation media, all isolates were named A to P in sequence. The number of recovered bacterial isolates was different between each of the five samples and five types of separation medium (Fig. 1) based on colony morphology. The coastal sediment harboured the most isolates with 90 bacteria (accounting for 36.14 % of all the separation microbes), followed by *P. trituberculatus* with 53 isolates (21.29 %), *O. oratoria* with 44 isolates (17.67 %), *M. veneriformis* with 35 isolates (14.06 %), and *L. vannamei* with 27 isolates (10.84 %). In comparison with the samples of four types seafood, more colonies forming on the plates were obtained from coastal sediment samples, which was consistent with the results of Nair et al. (2012), so that the sediment sample was selected for the separation of marine microorganisms in many studies (Annamalai et al. 2014).

The separation media used has a direct impact on the recoverability of microorganisms (Zhang et al. 2013). In this study, the separation medium of 2216E produced the most isolates (136) while NAC produced the fewest (16) accounting for 54.62 and 6.43 % of all separated isolates (249) respectively. APT, Mn^{2+} -NA, and TCBS produced 38, 32, and 27 isolates accounting for 15.26, 12.85, and 10.84 % respectively. 2216E was the best separation medium for bacterial recovery from coastal sediment and seafood samples, which may have been because 2216E is an exclusive medium for marine bacteria isolation and, when prepared with seawater containing a variety of metal ions and an appropriate salt, as opposed to distilled water, meant that 2216E was the closest match to the original growth environment of these marine microorganisms (Nair et al. 2012). The majority of microorganisms from the

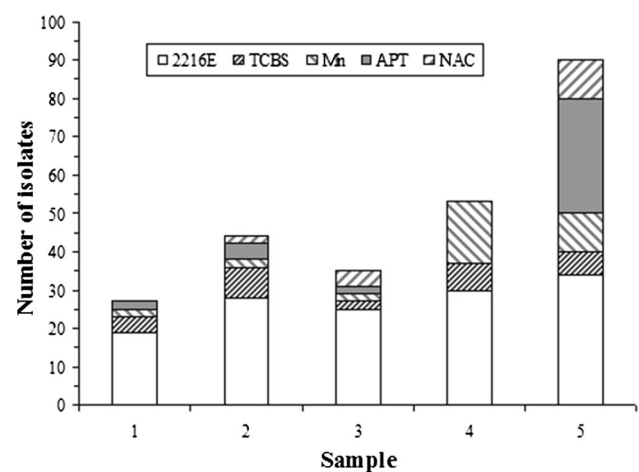


Fig. 1 Number of bacteria isolated from five samples using five separation media. Note: samples 1–4 were from the intestines or the gills of *L. vannamei*, *O. oratoria*, *M. veneriformis*, *P. trituberculatus*, respectively, sample 5 was from the coastal sediment

experimental samples came from a marine environment except for the sample from the gills of *O. oratoria*.

Direct antimicrobial activity of 249 isolates

All of the 249 bacterial isolates were screened for their direct antimicrobial activity against *V. parahaemolyticus* with spot inoculation. A total of 24 strains displayed antagonistic activity (Table 2) accounting for 9.64 % of the separation isolates. The direct antimicrobial activity assay for screening potential probiotic strains was performed on nutrient agar: if the strains needed special nutrients for producing antimicrobial metabolites, antagonism could not be observed.

Extracellular antimicrobial activity of the 16 selected isolates

Sixteen of 24 active isolates were selected for extracellular antimicrobial activity against *V. parahaemolyticus* using

the Oxford cup method. The other eight isolates (A2, A4, A8, A16, B15, F15, F16, and J9) were not studied any further for their poor inheritance stability (data not shown). The results of extracellular antimicrobial activity assays of 16 selected isolates are listed in Table 2. Among 16 selected active isolates, two strains (B16 and J7) displayed extracellular antimicrobial activity for the supernatant of B16 and J7 and the formation of inhibiting zones (Fig. 2). So strains of B16 and J7 were the most suitable candidate probiotics antagonistic to *V. parahaemolyticus* for sustainable aquaculture.

The other 14 strains (O8, O9, O12, O15, M1, L1, L5, L6, J2, J8, J11, J13, J18, and J21) with direct antimicrobial activity had no extracellular antimicrobial activity. Their supernatant could not form inhibiting zones. Interestingly, the culture of eight strains of O12, O15, M1, L1, L5, L6, J18, and J21 showed antagonism to *V. parahaemolyticus*. The reason for this result might be that the antimicrobial active ingredients of these eight strains were not

Table 2 The origin, separation medium, and inhibitory zone diameter (mm) of direct antimicrobial activity assay with spot inoculation and extracellular antimicrobial activity assay by Oxford cup method for 24 active isolates against *V. parahaemolyticus* in vitro

Active isolates	Origin	Separation medium	Direct antimicrobial activity	Extracellular antimicrobial activity	
				Culture	Supernatant
A2	Gills of <i>P. trituberculatus</i>	2216E	22.47 ± 0.7 ^a	N ^b	N
A4	Gills of <i>P. trituberculatus</i>	2216E	19.17 ± 0.68	N	N
A8	Gills of <i>P. trituberculatus</i>	2216E	18.27 ± 0.35	N	N
A16	Gills of <i>P. trituberculatus</i>	2216E	17.37 ± 1.02	N	N
B15	Gills of <i>O. oratoria</i>	2216E	15.65 ± 0.51	N	N
B16	Gills of <i>O. oratoria</i>	2216E	15.45 ± 0.29	14.97 ± 0.38	11.23 ± 0.39
F15	Intestines of <i>O. oratoria</i>	TCBS	14.09 ± 0.42	N	N
F16	Intestines of <i>O. oratoria</i>	TCBS	14.33 ± 0.94	N	N
O8	Coastal sediment	Mn ²⁺ -NA	18.23 ± 0.31	–	–
O9	Coastal sediment	Mn ²⁺ -NA	17.50 ± 0.20	–	–
O12	Coastal sediment	Mn ²⁺ -NA	22.17 ± 0.23	17.71 ± 0.19	–
O15	Coastal sediment	Mn ²⁺ -NA	17.77 ± 0.06	15.20 ± 0.17	–
M1	Intestines of <i>M. veneriformis</i>	NAC	20.72 ± 0.29	16.21 ± 0.20	–
L1	Intestines of <i>M. veneriformis</i>	Mn ²⁺ -NA	12.50 ± 0.26	16.09 ± 0.29	–
L5	Intestines of <i>M. veneriformis</i>	Mn ²⁺ -NA	16.00 ± 0.26	13.60 ± 0.09	–
L6	Intestines of <i>M. veneriformis</i>	Mn ²⁺ -NA	22.13 ± 0.06	18.21 ± 0.28	–
J2	Coastal sediment	APT	17.37 ± 0.15	–	–
J7	Coastal sediment	APT	23.63 ± 0.06	21.90 ± 0.19	20.10 ± 0.21
J8	Coastal sediment	APT	18.50 ± 0.10	–	–
J9	Coastal sediment	APT	22.51 ± 0.17	N	N
J11	Coastal sediment	APT	17.60 ± 0.10	–	–
J13	Coastal sediment	APT	18.60 ± 0.20	–	–
J18	Coastal sediment	APT	19.00 ± 0.10	11.91 ± 0.19	–
J21	Coastal sediment	APT	22.47 ± 0.21	18.01 ± 0.20	–

^a Mean ± SD, from triplicate determinations

^b Did not detect

– No inhibitory zone

extracellular secretions, so the cell-free supernatants had no antibacterial activity. In other relevant studies, the extracellular secretion of microorganisms was generally a source for novel antimicrobial substance extraction (Snook et al. 2009; Bacon et al. 2012). However, from the results of this study, the effective antibacterial ingredients of these eight isolates should be in their own cellular material. Those with the potential to become probiotics in aquaculture would probably be missed if the extracellular secretions of microorganisms was the research target. We do not know anything about the chemical properties of the antimicrobial compound(s) of any isolated strains in this study, so further research should focus on the chemical nature and the biosecurity of antimicrobial ingredients produced by such isolates to provide a theoretical basis for further fundamental research and application in aquaculture.

Identification of potential probiotics B16 and J7

Preliminary morphological and physiological identifications

The colony morphology of strain B16 was a light yellow, flat, glossy surface; while strain J7 was white, opaque, with a rough surface, and irregular edges around the colony on nutrient agar. Two potential probiotics were both Gram-positive, *Bacillus* spp. The spores of strains B16 and J7 were located in the middle of the cell, and underwent no swelling. Differences between biochemical tests of the two strains included the ability to use xylose, lysine, amylum, and nitrate. Biochemical test results are listed in Table 3. Based on the morphological, cultural, and physiological

characteristics, strains B16 and J7 should be members of the *Bacillus subtilis* group.

Molecular biological identifications based on 16S rRNA gene sequences

The length of the 16S rRNA sequences of the strains of B16 and J7 was around 1450 bp as detected by 1 % agarose gel electrophoresis (data not shown). The sequences of two potential probiotics and 16S rRNA sequences obtained from other species within the same genus, and downloaded from GenBank at NCBI, were aligned to construct the phylogenetic tree. The results indicated that two the potential probiotic strains of B16 and J7 were closely related to *Bacillus* spp. which is well known for having the widely antimicrobial activity (Aunpad and Na-Bangchang 2007; Aunpad and Panbangred 2012; Li et al. 2012; Xu et al. 2014).

The identities of 16S rRNA between strains of B16 and *Bacillus pumilus* ATCC 7061 and strains of J7 and *Bacillus mojavensis* SWFU16 were 100 and 99 % respectively (Fig. 3). Two strains with a sequence identity of greater than 99 % should be classified as the same species according to Wu and Ahn (2011). It is difficult for *B. pumilus* and *B. mojavensis*, having a *B. subtilis*-like phenotype, to be distinguished from *B. subtilis*. Therefore, the 16S rRNA sequences analysis was used to determine the phylogenetic status for these potential probiotic strains of B16 and J7, which is the basis for identifying this kind of species (Roberts et al. 1994) and classifying bacteria (Woese 1987). Based on the results of physiological tests and 16S rRNA sequence analysis, strains B16 and J7 were identified as *B. pumilus* and *B. mojavensis*, respectively. As far as we

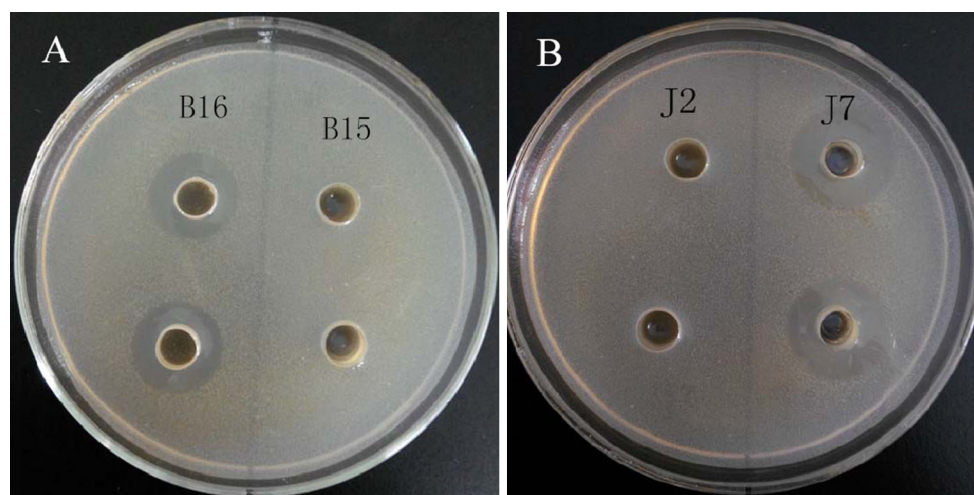


Fig. 2 The antagonistic activity of the supernatant of potential probiotics B16 (A) and J7 (B) against *V. parahaemolyticus* in vitro. Note The left two holes were the supernatant of isolate B16 (A) and J2

(B), the right two holes were the supernatant of isolate B15 (A) and J7 (B). Each isolate of the third replica was in another plate

know, this is the first report of *B. mojavensis* being isolated from Liaodong Bay.

Enzymatic activities of B. pumilus B16 and B. mojavensis J7

Both *B. pumilus* B16 and *B. mojavensis* J7 exhibited proteinase activity and lipase activity. As for amylase activity *B. mojavensis* J7 was positive while *B. pumilus* B16 was negative. *B. pumilus* B16 and *B. mojavensis* J7 had the production capacity of extracellular hydrolytic enzymes, which made them potential probiotic candidates for the enhancement of digestion and nutrient uptake by the host (Nimrat et al. 2011). Members of *Bacillus*, compared with other species of probiotics, have achieved considerable success by the use of endospore-forming agents (Hong et al. 2005), the screened *B. pumilus* B16 and *B. mojavensis* J7 in this study provided new probiotic candidates.

Antagonistic spectrum of B. pumilus B16 and B. mojavensis J7

The culture and supernatant of *B. pumilus* B16 and *B. mojavensis* J7 were further investigated against the other ten pathogens (three Gram-positive bacteria and seven Gram-negative bacteria) by Oxford cup method. Five among the 10 indicator bacteria were common pathogens in aquaculture, others were human pathogens. The supernatants of *B. pumilus* B16 and *B. mojavensis* J7 showed extracellular antimicrobial activity to more than one indicator bacteria in this research. Three indicator bacteria were inhibited by the supernatant of *B. pumilus* B16 including *S. typhimurium*, *L. monocytogenes* and *S. aureus*. Moreover, four indicator bacteria were inhibited by the supernatant of *B. mojavensis* J7 including *V. vulnificus*, *V. harveyi*, *E. coli*, and *S. aureus* (Table 4). *Staphylococcus aureus* was the most sensitive indicator, while the indicator

Table 3 The results of biochemical tests of two potential probiotic strains (B16 and J7) isolated in this study

Characteristics	B16	J7	Characteristics	B16	J7	Characteristics	B16	J7
Glucose ^a	+	+	Nitrate reduction	-	+	2 %/5 %/7 % NaCl	+	+
Xylose	-	+	Amylolysis	-	+	Glucose aerogenesis	-	-
Motility	+	+	Trehalose	+	+	Anaerobic growth	-	-
Arabinose	+	+	pH 5/6/7	+	+	Tyrosine hydrolysis	-	-
Sucrose	+	+	Mannitol	+	+	Utilization of citrate	+	+
Raffinose	-	-	Sorbitol	+	+	Egg yolk reaction	-	-
Adonitol	-	-	V-P	+	+	Contact enzyme reaction	+	+
Lysine	-	+	Indole reaction	-	-	Growth temperature 5/55 °C	+	+

^a The biochemical characterisation of potential probiotics B16 and J7 were based on sugar fermentation pattern using different carbohydrates as a source of carbon in basal broth medium for their ability to ferment different sugars

+ Positive reaction, - Negative reaction

Fig. 3 Neighbour-joining tree exhibiting the phylogenetic positions of isolate B16, J7, and representatives of other related strains within *Bacillus* genus based on 16S rDNA gene sequences

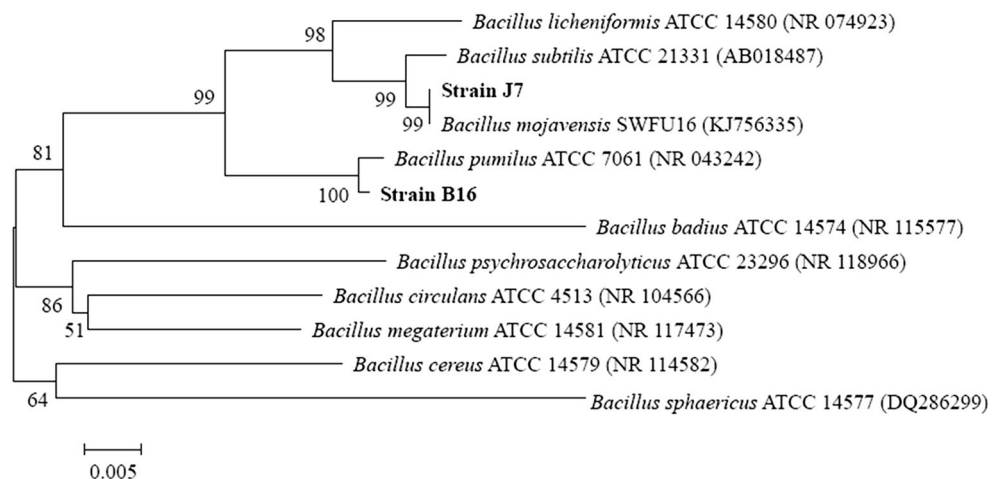


Table 4 The antagonistic spectra of potential probiotics (*B. pumilus* B16 and *B. mojavensis* J7) isolated in this study with Oxford cup method (All values are in mm.)

Indicator strains	<i>B. pumilus</i> B16		<i>B. mojavensis</i> J7	
	Culture	Supernatant	Culture	Supernatant
<i>V. parahaemolyticus</i> ATCC 33847	9.53 ± 0.18 ^a	–	12.43 ± 0.14	–
<i>V. vulnificus</i>	11.13 ± 0.61	–	13.05 ± 0.39	11.45 ± 0.33
<i>V. harveyi</i>	–	–	11.68 ± 0.56	11.45 ± 0.77
<i>S. typhimurium</i>	9.37 ± 0.49	7.91 ± 0.38	10.24 ± 0.33	–
<i>P. aeruginosa</i>	–	–	16.01 ± 0.52	–
<i>A. hydrophila</i>	12.71 ± 0.16	–	–	–
<i>E. coli</i>	–	–	11.28 ± 0.26	10.31 ± 0.26
<i>L. monocytogenes</i>	12.87 ± 0.64	10.94 ± 0.37	11.11 ± 0.45	–
<i>B. subtilis</i>	10.05 ± 0.36	–	–	–
<i>S. aureus</i>	10.47 ± 0.38	10.03 ± 0.46	12.33 ± 0.45	10.82 ± 0.35

^a The inhibitory zone diameters (Mean ± SD, from triplicate determinations)

– No inhibitory zone

bacteria of *V. parahaemolyticus* ATCC 33847, *P. aeruginosa*, *A. hydrophila*, and *B. subtilis* were not inhibited by the supernatants of *B. pumilus* B16 and *B. mojavensis* J7 in this study.

There is no particular trait as to antagonism of *B. pumilus* B16 or *B. mojavensis* J7 to Gram-positive, or Gram-negative, indicator bacteria. Combined with the result of this extracellular antimicrobial activity assay, the culture and/or the isolated cell itself may contain some kind of antimicrobial compounds which did not exist in the supernatant. It has been reported that even dead, or inactivated, probiotic cells had the ability to stimulate the innate immune system of hydrocoles (Irianto and Austin 2003). So the culture and/or isolated cells with direct antimicrobial activity should be used as the source for novel antimicrobial substance extraction (Lai and Wong 2013). Other research confirmed that members of genus *Bacillus* can produce more than one type of antimicrobial metabolite (Aunpad and Panbangred 2012; Bacon et al. 2012; Li et al. 2012; Xu et al. 2014). In addition, the strain of *B. pumilus* B16 or *B. mojavensis* J7 may produce different types, or amounts, of antimicrobial agents according to the variation in the potency of antagonism to different indicator bacteria (Table 4). This was in accordance with the result of Vynne et al. (2011) that the strain could change secondary metabolite production to adjust inhibitory activity within the species level itself. To our knowledge, this is the first time that reported that *B. mojavensis* can inhibit pathogenic *Vibrio* spp., including *V. parahaemolyticus* (ATCC 17802), *V. vulnificus* (environmentally isolated) and *V. harveyi* (environmentally isolated).

In recent years, some studies of the antagonistic activity of the isolated bacteria have been undertaken, and a variety of organisms antagonistic to pathogens in aquaculture have been found, including *B. pumilus* B16 and *B. mojavensis* J7 in this study. Due to *Bacillus* having the ability to produce heat-resistant spores, they have good prospects for application as probiotics.

Conclusion

In this study, the isolation, screening, and identification of *Bacillus* spp. antagonistic to *V. parahaemolyticus* were reported. The *Bacillus* spp. respectively isolated from the coastal sediment and the gills of *O. oratoria* showed biological control potential in aquaculture. The culture and/or the isolated cell, with its steady, direct, antimicrobial activity, could be used as a source for the exploration of novel bio-control agents. Further research will continue on the purification, identification, and biosecurity of antibacterial metabolites produced by those potential probiotics *B. pumilus* B16 and *B. mojavensis* J7 obtained here.

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References

- Annamalai N, Rajeswari MV, Sahu SK, Balasubramanian T (2014) Purification and characterization of solvent stable, alkaline protease from *Bacillus firmus* CAS7 by microbial conversion of marine wastes and molecular mechanism underlying solvent stability. *Process Biochem* 49:1012–1019
- Aranda CP, Valenzuela C, Barrientos J, Paredes J, Leal P, Maldonado M, Godoy FA, Osorio CG (2012) Bacteriostatic anti-*Vibrio parahaemolyticus* activity of *Pseudoalteromonas* sp. strains DIT09, DIT44 and DIT46 isolated from Southern Chilean intertidal *Perumytilus purpuratus*. *World J Microbiol Biotechnol* 28:2365–2374
- Aunpad R, Na-Bangchang K (2007) Pumilicin 4, a novel bacteriocin with anti-MRSA and anti-VRE activity produced by newly isolated bacteria *Bacillus pumilus* strain WAPB4. *Curr Microbiol* 55:308–313
- Aunpad R, Panbangred W (2012) Evidence for two putative holin-like peptides encoding genes of *Bacillus pumilus* strain WAPB4. *Curr Microbiol* 64:343–348

- Bacon CW, Hinton DM (2002) Endophytic and biological control potential of *Bacillus mojavensis* and related species. *Biol Control* 23:274–284
- Bacon CW, Hinton DM, Mitchell TR, Snook ME, Olubajo B (2012) Characterization of endophytic strains of *Bacillus mojavensis* and their production of surfactin isomers. *Biol Control* 62:1–9
- Barman P, Banerjee A, Bandyopadhyay P, Chandra MK, Mohapatra PKD (2011) Isolation, identification and molecular characterization of potential probiotic bacterium, *Bacillus subtilis* PPP 13 from *Penaeus monodon*. *Biotechnol Bioinform Bioeng* 1:473–482
- Chahad OB, El Bour M, Calo-Mata P, Boudabous A, Barros-Velázquez J (2012) Discovery of novel biopreservation agents with inhibitory effects on growth of food-borne pathogens and their application to seafood products. *Res Microbiol* 163:44–54
- Donio MBS, Velmurugan S, Raman K, Babu MM, Citarasu T (2014) Antagonistic *Bacillus cereus* TC-1 isolated from solar salt work in southern India. *J Microb Biochem Technol* 6:242–246
- Fernandez-Piquer J, Bowman JP, Ross T, Tamplin ML (2011) Predictive models for the effect of storage temperature on *Vibrio parahaemolyticus* viability and counts of total viable bacteria in Pacific oysters (*Crassostrea gigas*). *Appl Environ Microbiol* 77:8687–8695
- Fuller R (1989) Probiotics in man and animals. *J Appl Bacteriol* 66:365–378
- Griffitt KJ, Noriega NF III, Johnson CN, Grimes DJ (2011) Enumeration of *Vibrio parahaemolyticus* in the viable but nonculturable state using direct plate counts and recognition of individual gene fluorescence in situ hybridization. *J Microbiol Methods* 85:114–118
- Hong HA, Duc LH, Cutting SM (2005) The use of bacterial spore formers as probiotics. *FEMS Microbiol Rev* 29:813–835
- Irianto A, Austin B (2003) Use of dead probiotic cells to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis* 26:59–62
- Iwamoto M, Ayers T, Mahon BE, Swerdlow DL (2010) Epidemiology of seafood-associated infections in the United States. *Clin Microbiol Rev* 23:399–411
- Lai WB, Wong HC (2013) Influence of combinations of sublethal stresses on the control of *Vibrio parahaemolyticus* and its cellular oxidative response. *Food Control* 33:186–192
- Li GG, Liu BS, Shang YJ, Yu ZQ, Zhang RJ (2012) Novel activity evaluation and subsequent partial purification of antimicrobial peptides produced by *Bacillus subtilis* LFB112. *Ann Microbiol* 62:667–674
- Nair AV, Vijayan KK, Chakrabort K, Antony ML (2012) Diversity and characterization of antagonistic bacteria from tropical estuarine habitats of Cochin, India for fish health management. *World J Microbiol Biotechnol* 28:2581–2592
- Newaj-Fyzul A, Al-Harbi AH, Austin B (2014) Review: developments in the use of probiotics for disease control in aquaculture. *Aquaculture* 431:1–11
- Nimrat S, Boonthai T, Vuthiphandchai V (2011) Effects of probiotic forms, compositions of and mode of probiotic administration on rearing of Pacific white shrimp (*Litopenaeus vannamei*) larvae and postlarvae. *Anim Feed Sci Technol* 169:244–258
- Nithya C, Aravindraj C, Pandian SK (2010) *Bacillus pumilus* of Palk Bay origin inhibits quorum-sensing-mediated virulence factors in Gram-negative bacteria. *Res Microbiol* 161:293–304
- Raghunath P, Acharya S, Bhanumathi A, Iddya Karunasagar, Indrani Karunasagar (2008) Detection and molecular characterization of *Vibrio parahaemolyticus* isolated from seafood harvested along the southwest coast of India. *Food Microbiol* 25:824–830
- Ravi AV, Musthafa KS, Jegathambal G, Kathiresan K, Pandian SK (2007) Screening and evaluation of probiotics as a biocontrol agent against pathogenic *Vibrios* in marine aquaculture. *Lett Appl Microbiol* 45:219–223
- Roberts MS, Nakamura LK, Cohan FM (1994) *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequence, and differences in fatty acid composition. *Int J Syst Bacteriol* 44:256–264
- Salminen S, Ouwehand A, Benno Y, Lee YK (1999) Probiotics: How should they be defined? *Trends Food Sci Technol* 10:107–110
- Snook ME, Mitchell T, Hinton DM, Bacon CW (2009) Isolation and characterization of Leu7-surfactin from the endophytic bacterium *Bacillus mojavensis* RRC 101, a biocontrol agent for *Fusarium verticillioides*. *J Agric Food Chem* 57:4287–4292
- Touraki M, Karamanlidou G, Karavida P, Chrysi K (2012) Evaluation of the probiotics *Bacillus subtilis* and *Lactobacillus plantarum* bioencapsulated in *Artemia* nauplii against vibriosis in European sea bass larvae (*Dicentrarchus labrax*, L.). *World J Microbiol Biotechnol* 28:2425–2433
- Vynne NG, Månsson M, Nielsen KF, Gram L (2011) Bioactivity, chemical profiling, and 16S rRNA-based phylogeny of *Pseudomonas* strains collected on a global research cruise. *Mar Biotechnol* 13:1062–1073
- Woese CR (1987) Bacterial evolution. *Microbiol Rev* 51:221–271
- World Health Organization, Food Agriculture Organization of the United Nations (2011) Risk assessment of *Vibrio parahaemolyticus* in seafood: interpretative summary and technical report
- Wu WJ, Ahn BY (2011) Isolation and identification of *Bacillus amyloliquefaciens* BY01 with high productivity of menaquinone for *Cheonggukjang* production. *J Korean Soc Appl Biol Chem* 54:783–789
- Wu YN, Wen J, Ma Y, Ma XC, Chen Y (2014) Epidemiology of foodborne disease outbreaks caused by *Vibrio parahaemolyticus*, China, 2003–2008. *Food Control* 46:197–202
- Xu P, Zeng YC, Fong Q, Lone T, Liu YY (2012) Chinese consumers' willingness to pay for green- and eco-labeled seafood. *Food Control* 28:74–82
- Xu HM, Rong YJ, Zhao MX, Song B, Chi ZM (2014) Antibacterial activity of the lipopeptides produced by *Bacillus amyloliquefaciens* M1 against multidrug-resistant *Vibrio* spp. isolated from diseased marine animals. *Appl Microbiol Biotechnol* 98:127–136
- Yu WT, Jong KJ, Lin YR, Tsai SE, Tey YH, Wong HC (2013) Prevalence of *Vibrio parahaemolyticus* in oyster and clam culturing environments in Taiwan. *Int J Food Microbiol* 160:185–192
- Zarei M, Borujeni MP, Jamnejad A, Khezzadeh M (2012) Seasonal prevalence of *Vibrio* species in retail shrimps with an emphasis on *Vibrio parahaemolyticus*. *Food Control* 25:107–109
- Zhang XY, He F, Wang GH, Bao J, Xu XY, Qi SH (2013) Diversity and antibacterial activity of culturable actinobacteria isolated from five species of the South China Sea gorgonian corals. *World J Microbiol Biotechnol* 29:1107–1116
- Zokaeifar H, Balcázar JL, Saad CR, Kamarudin MS, Sijam K, Arshad A, Nejat N (2012) Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol* 33:683–689