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Original Article

Characterization of a probiotic *Bacillus* S11 bacterium of black tiger shrimp *Penaeus monodon*

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Abstract

Bacillus S11 (BS11), a Gram-positive spore forming bacteria, was identified as *Bacillus subtilis*, based on biochemical tests, physical morphology, and 16S rRNA gene sequence. BS11 was found to be safe as probiotic for shrimp because it does not produce either detectable antimicrobial substance or enterotoxin. A potential specific markers of BS11 by RAPD-PCR was indicated using UBC459 primer (5'-GCGTCGAGGG -3') and the sequence of the major band, a size of 0.4 kb fragment, is similar to the gene encoding of a protein of the phosphotransferase system (PTS) glucosamine-specific enzyme glucosamine-6-phosphate isomerase from *Bacillus subtilis* strain subsp. *subtilis* 168.

Keywords: *Bacillus subtilis*, BS11, black tiger shrimp, probiotic bacterium, *Penaeus monodon*

1. Introduction

Probiotics have already become a significant direction as an alternative to antibiotic treatment for aquaculture and have been commercially available as feed or water additives in pond water (Moriarty, 1997; Boyd and Gross, 1998; Verschuere *et al*., 2000; Wang *et al*., 2005). Recently, FAO has designated the use of probiotics as a major agent for the improvement of aquatic environmental quality (Subasinghe *et al*., 2003). The term, probiotic, originating from the Greek words "pro" and "bios" means "for life"," and was firstly created by Lilley and Stillwell in 1965 as "the substances secreted by one microorganism, which stimulated the growth of another". Later, the definition of probiotic has been gradually changed and could be combined and referred to as "living microorganism mono- or mixed culture in sufficient number with or without by-products, leading to benefit of host health by improving intestinal microbial balance and of environment (Sperti, 1971; Parker, 1974; Fuller, 1992 and 1997; Havenaar and Huis In't Veld, 1992; Salminen, 1996; Schaafsma, 1996; Gatesoupe, 1999; Tannock, 1999; Gismondo *et al*., 1999; Verschuere *et al*., 2000; FAO, 2001; Irianto and Austin, 2002). Among probiotic bacteria for shrimp *Bacillus* spp. are more widely used and proved to enhance shrimp health with no visible side effects (Vaseeharan, 2003; Ziaei-Nejad *et al*., 2006; Balcázar, and Rojas-Luna, 2007; Gomez and Shen, 2008; Tseng *et al*., 2009; Liu *et al*., 2010). *Bacillus* probiotic supplement in shrimp feed (Rengpipat *et al*., 1998; Balcázar, 2003; Tseng, 2009) or culture water (Moriarty, 1998; Gullian *et al*., 2004; Ziaei-Nejad, 2006) is expanding rapidly with an increasing number of studies demonstrating immune stimulation, antimicrobial activities, and competitive exclusion. Recently, the implement of *Bacillus subtilis* and *B. indicus* have been approved for use as a human food supple-

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ment in Italy, as well as *B. clausii* is licensed as a prophylactic medicine in the product "Enterogermina" (Duc *et al*., 2004; Cutting, 2010). Moreover, food like natto of Japan is comprised of safe *Bacillus* spp. (Ueda, 1989).

Bacillus sp. (BS11) was isolated from the gastrointestinal tract of black tiger shrimp *Penaeus monodon* and has been proved to enhance shrimp health with no visible side effects on shrimp or culture water after conducting a trial on shrimp (Rengpipat *et al*., 1998, 2000, 2003). BS11 has never been harmful to the host. It can pass through ingestion and it has been accepted by shrimp including the ability to locate and colonize on the GI tract surface (Rengpipat, 2005; Rengpipat *et al*., 2009). BS11 can increase their number during they adhere on the shrimp intestine's surface. The ability to compete and inhibit pathogens *in vivo* and *in vitro*, respectively, have also been studied (Phianphak, 1996). From its properties BS11 clearly possess probiotic properties that can be utilized for black tiger shrimp. In this study BS11 has been extensively studied and characterized to ensure its safety as probiotic for shrimp.

2. Materials and Methods

2.1 Bacterial strain

Bacillus S11 (BS11), a probiotic of black tiger shrimp *Penaeus monodon* as reported by Rengpipat *et al.* (1998), was grown in Trypticase Soy broth (TSB; Difco, Sparks, MD, USA) and maintained on Trypticase Soy agar (TSA; Difco, Sparks, MD, USA) at 30°C. Bacteria pellet were washed twice with sterile 0.15 M phosphate buffered saline pH 7.2 (PBS), re-suspended in PBS. The absorbance of the final bacterial suspensions were adjusted to 1.0 at 660 nm using PBS. Suspension of bacterial cells was kept in glycerol (20%) with equal volume at -70°C for further use.

2.2 BS11 identification

The identification of BS11 was carried out using both conventional methods and the test kit of api® 20E and api® 50 CHB Medium, bioMérieux, Marcy-I'Etoile, France. The results were analyzed with the APILAB Plus software. The organism was confirmed and regularly checked their purity by following conventional methods, for example, Gram staining, spore staining, oxidase and catalase tests. Their morphology was investigated under microscopic examination (Figure 1) and on agar plate. Physiological characteristics were determined in TSB with various concentrations of salt (NaCl), with different pH, and temperature. All medium and chemical solutions were provided by Difco, Sparks, MD, USA.

2.3 Determination of nucleotide sequence of 16S rDNA

Genomic DNA was extracted by Genome DNA Simax Kit (Beijing SBS Genetech Co., Ltd., China). The PCR amplification was carried out in a DNA thermo cycler TP 600 (TaKaRa Bio Inc., Otsu, Shiga, Japan) with universal primers: 16F27 (5'-AGA GTT TGA TCC TGG CTC AG-3') and 16R1522 (5'-AAG GAG GTG ATC CAG CCG CA-3'). The method was used according to the manufacturer's instructions. The PCR conditions consisted of 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. Nucleotide sequencing was commercially serviced by Macrogen Inc. co. Ltd. (Seoul, Korea). The obtained 16S rDNA sequence was compared with those in the database and the phylogenic tree was made using Neighbor Joining tree, PHYLIP Version 3.5 (see http://evolution.genetics.washington.edu/phylip.html, accessed July 23, 2009).

2.4 Isolation of chromosomal DNA

Chromosomal DNA was prepared from an 18 hrs-culture at 30°C of BS11 in Trypticase Soy broth (TSB). Cells were harvested after centrifugation at 9,820 g for 3 min and suspended in 500 µl of 50 mM Tris-HCl, pH 8.0, 5mM (TE buffer). After freezing at -80°C and thawing cells at 100°C, each of 5 min for five times, suspension was re–centrifuged at 25,931 g for 10 min. The supernatant was added with 1x volume of phenol chloroform for DNA extraction. The aqueous upper layer was transferred into fresh tube and 1/10x volume of 3M Na-acetate and absolute ethanol 2x volume were added and kept at -20°C for 1 h. DNA was precipitated by centrifugation at 25,931 g for 10 min followed by washing with 70% (v/v) ethanol, and centrifuged at 25,931 g for 10 min. DNA pellet were dried in heat box at 50°C, re-suspended in 50 µl TE buffer containing 1 µl of RNase (10

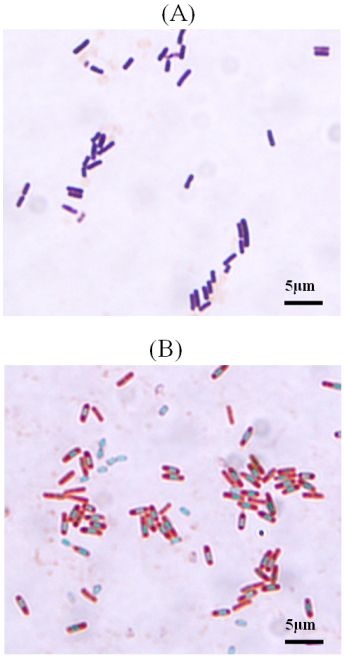

Figure 1. Gram-positive rods of BS11, the size of ~0.45-0.55 x 2.5-3.5 (width x length) µm (A); central endospore and free spore in green of BS11 (B), under light microscope (X1000) (Olympus BX51, Japan).

mg/ml), and incubated at 37°C for 1 h. DNA suspension was kept at -20°C before use.

2.5 RAPD-PCR analysis

RAPD-PCR (Randomly Amplified Polymorphic DNA-PCR) was performed in a 25 ml reaction mixture containing 1X buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 4 mM $MgCl_2$, 100 µM of each dATP, dCTP, dTTP and dGTP (SibEnzyme, Academtown, Russia), 0.4 mM of an arbitrary primer: OPA01(5'-CAGGCCCTTC-3'), OPA02(5'-TGCCGAGCTG-3'), OPB10(5'-CTGCTGGGAC-3') (Eurofins MWG Operon, Alabama, U.S.A.); UBC122(5'-GTAGACGAGC -3'), UBC428(5'-GGCTGCGGTA-3'), UBC459(5'-GCGTCGAG GG -3') (University of British Columbia, Canada); 25 ng of genomic DNA and 1 unit of DyNazyme™ II DNA polymerase (Finnzymes, Finland). PCR was performed using GeneAmp 2400 PCR system (Perkin Elmer, Waltham, USA) and amplification conditions included an initial denaturation step at 94°C for 3 min, 40 cycles of 94°C for 15 s, 36°C for 60 s, 72°C for 90 s, and final extension at 72°C for 7 min. Five microliters of the amplification reaction were electrophoresed through 1.5% w/v agarose gel with a molecular size marker (100bp DNA) ladder (SibEnzyme, Academtown, Russia) and visualized by a UV transilluminator after ethidium bromide staining.

2.6 Cloning and sequencing of BS11-specific RAPD fragments

RAPD fragments found only in a particular species were eluted out from agarose gel using a HiYield ™ Gel/PCR extraction kit (Real Biotech Corporation, Taipei, Taiwan) and ligated to pGEM®-T easy vector (Promega, Madison, Wisconsin, U.S.A.) according to protocols recommended by the manufacturer's instruction and described previously (Klinbunga *et al*., 2004). The ligation product was transformed to *E. coli* JM109. Recombinant clones were identified by colony PCR. White colonies on LB gar plate containing ampicillin (50 ug/ml), IPTG (25 ng/µl) and X-Gal (25 ng/µl) were picked and restreaked for confirmation of transformants. Plasmid DNA was extracted from the overnight culture using a HiYield™ plasmid mini kit (Real Biotech Corporation, Taipei, Taiwan) and unidirectional sequenced by an automated DNA sequencer (ABI310) at the Bioservice unit (BSU), National Center for Genetic Engineering and Biotechnology (BIOTEC), Bangkok, Thailand. The sequence was compared with sequences in the database using NCBI's BLAST program.

Bacillus subtilis ATCC 6633, *Bacillus thuringiensis* IAM 11064, *Bacillus sphaericus* IAM 13420, *Bacillus amyloliquefaciens* IAM 1521, *Bacillus coagulans* IAM 1115, *Bacillus subtilis* (provided by Department of Microbiology, Faculty of Science, Chulalongkorn University) and BP 11 (preliminary identified as *Bacillus subtilis* from our research group), were cultivated in TSB at 30°C for 24 hrs. Their chromosomal DNA was prepared by following the same procedure as mentioned above.

2.7 Determination of antimicrobial substance and enterotoxin

Antimicrobial substance from BS11 was preliminarily determined using Antimicrobial residue screening test kit (AM-Test) developed by a research group at the Center for Antimicrobial Resistance Monitoring in Food-borne Pathogens (in collaborating with WHO), Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. The principle was based on tube diffusion method and detection limit for 18 antibiotics were in the range of >0.001-0.1 ppm which depended on antibiotic type. Filtrate of 0.1 ml from the BS11- cultured TSB broth after 24 hrs and 48 hrs incubation at 30°C (spore forming bacillus) was passed through a sterile filter (0.22 µm) and was then transferred into tube containing semi -solid agar with spores of *Geobacillus stearothermophilus* and pH indicator. Milk containing antibiotic was used as positive control. Tubes were incubated at 65±1°C for 2-2.5 hrs. During incubation the antibiotic in the sample would diffuse into agar and inhibit *Geobacillus stearothermophilus* growth until purple color remained. If there is no antibiotic in the milk yellow color would be detected.

Determination of enterotoxin from filtrates of BS11-cultured broth were performed by following the procedures according to manufacturer's instructions (TECRA® test kit for the detection of Bacillus Diarrhoeal Enterotoxin (BDE); TECRA International Pty Ltd, Frenchs Forest NSW 2086, Australia). This enterotoxin test was based on "sandwich" configuration of Enzyme-linked immunosorbent assay (ELISA).

3. Results and Discussion

3.1 BS11 identification and its characteristics

BS11 is a Gram-positive, rod-shaped bacteria with the cell size of ~0.45-0.55 x 2.5-3.5 µm (width x length) and can form central spore as shown in Figure 1A and B, respectively. Biochemical characteristics of BS11 were identified using the test kits of api® 20E and api® 50 CHB. Medium and physiological properties examined using microbiological methods were indicated in Table 1. After analysis with the APILAB Plus software, percentages of similarity of BS11 to *Bacillus subtilis* is 99.9%. Genetic analysis using 16S rRNA gene was performed to identify BS11. The sequence of 16S rDNA from BS11 shows close relatedness with *Bacillus subtilis*, and its sequence (1513 base pair) was submitted to NCBI gene bank and obtained the accession number of the Genbank, GU 166746.

Phylogenetic tree showed close similarity of BS11 with *Bacillus subtilis* (Figure 2). Results of the biochemical analysis were in agreement of 16S r-RNA analysis for BS11. Therefore, BS11 is mostly classified as *Bacillus subtilis*.

In order to identify the specific markers of BS11, all *Bacillus* spp. were analyzed by RAPD-PCR using independently prepared template DNAs in triplicate. Among six

Table 1. Biochemical tests and characterization for BS11 using the api® 20E and api® 50 CHB Medium test kits and microbiological techniques (+ = positive, - = negative).

Biochemical tests	BS11	Biochemical tests	BS11
Control	-	Starch	-
Glycerol	+	Glycogen	-
Erythritol	-	Xylitol	-
D-arabinose	-	Gentiobiose	-
L-arabinose	+	D -turanose	+
D-ribose	+	D -lyxose	-
D-xylose	+	D-tagatose	-
L-xylose	-	D -fucose	-
D-adonitol	-	L -fucose	-
Methyl-βD-Xylopyranoside	-	D -arabitol	-
D-galactose	-	L -arabitol	-
D-glucose	+	Gluconate	-
Fructose	+	2-keto-gluconate	-
D-mannose	+	5-keto-gluconate	-
L-sorbose	-	Beta-galactosidase	+
L-rhamnose	-	Arginine dihydrolase	-
Dulcitol	-	Lysine decarboxylase	-
Inositol	+	Ornithine decarboxylase	-
D-mannitol	+	Citrate	+
D-sorbitol	+	Hydrogen sulfide	-
Methyl-αD-Mannopyranoside	-	Urease	-
Methyl-αD-Glucopyranoside	+	Trytophane deaminase	-
N-acetylglucosamine	-	Indole	-
Amygdalin	+	VP	+
Arbutin	+	Gelatinase	+
Esculin	+	Nitrate reduction	+
Salicin	+	O-F test	oxidation
D-cellobiose	+	Catalase	+
D-maltose	+	Caseinase	+
D-lactose(bovine origin)	-	Amylase	+
D-melibiose	+	Lipase	+
D-saccharose(sucrose)	+	Nuclease	-
D-trehalose	+	Range of growth	
Inulin	-	Temperature 20-45°C	+
D-melezitose	-	pH 5-8	+
D-raffinose	+	NaCl >0.1-8% (w/v)	+

primers only UBC459 primer (5'-GCGTCGAGGG -3') was selected because it generated distinctive bands with BS11 as shown in Figure 3, which made it possible to distinguish BS11 from the other *Bacillus* spp.. The major band of BS11 a size of 0.4 kb fragment was gel extracted and sequenced. From NCBI'Blast analysis, this sequence is similar to the gene encoding a hypothetical protein of the phosphotransferase system (PTS) glucosamine-specific enzyme glucosamine-6-phosphate isomerase from *Bacillus subtilis* subsp. subtilis strain 168. This UBC459 primer and RAPD-PCR protocol should be used to identify BS11 accurately.

3.2 Antimicrobial and enterotoxin detection

Yellow color detection in AM-test after tubes filled with filtrates of BS11-cultured broth, which indicated that no antimicrobial residue producing from BS11 was found; whereas, milk containing antibiotic as positive control showed purple color. In addition no *Bacillus* diarrhoeal enterotoxin detected in BS11-cultured broth after tested by following procedures of TECRA test kit. Only green color on the paper strip was seen after positive diarrhoeal enterotoxin control provided by manufacturer was added.

Figure 2. Phylogenetic relationships of BS11 and *Bacillus subtilis*, *B. licheniformis, B. pumilus, B. halodurans, B. mycoides, Halobacillus ritolaris*, *H. salinus, Sporosarcina halophila*, *and Escherichia coli* as an outgroup were inferred from the alignment of the 1513 bp of 16S rRNA coding region, using Neighbor Joining tree PHYLIP Version 3.5. Bootstap values given for each node greater than 70% were considered significant. The bar represents the unit length of the number of nucleotide substitutions per site.

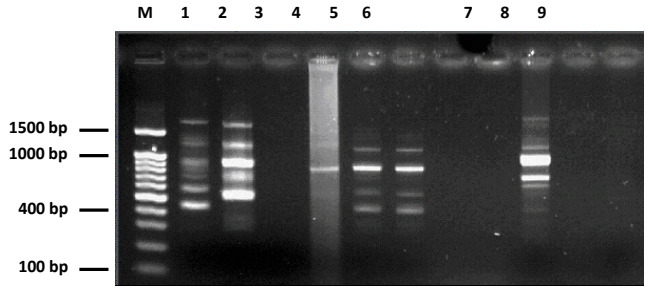

Figure 3. PCR using UBC 459 Primer. RAPD-PCR patterns of BS11 and reference strains:1, BS11; 2, BP11; 3, *Bacillus thuringiensis* IAM 11064; 4, *Bacillus sphaericus* IAM 13420; 5, *Bacillus amyloliquefaciens* IAM 1521; 6, *Bacillus coagulans* IAM 1115; 7, *Bacillus subtilis* ATCC 6633; 8, *Bacillus subtilis*; 9, negative control (sterile distilled water instead of DNA sample was used); M, 100 bp size ladder (SibEnzyme, Academtown, Russia). 1.5% agarose gel was used for the electrophoresis.

Bacillus subtilis, a saprophytic Gram-positive, spore-forming bacteria, is not a human or animal pathogen, nor is it toxigenic like the other members of this genus (U.S. EPA, 1997). In addition, this species is known to be non- or low-virulent and requires very high number of bacteria for causing disease in human (Edberg, 1991). BS11, identified as *Bacillus subtilis*, is a major flora of black tiger shrimp's gastrointestinal tract and possesses probiotic properties for shrimp via feed additive (Phianphak, 1996; Rengpipat *et al.*, 1998, 2000, 2003, 2009). BS11 may firstly inhabit in soil or sediment at the bottom of shrimp pond. Since generally the main habitat of *B. subtilis* is soil, sediments, air, dust, water, and decomposing plant residues (Alexander, 1977; Moriarty, 1999; Gatesoupe, 1999; Green *et al*., 1999). BS11 can survive in media with various pH, temperatures, and salt concentrations (Table 1). BS11 also showed ability to produce spores only in TSA after 48 hrs of culture (Figure 1B), but never be detected from shrimp larvae or water samples after heat shock at 80°C for 10 min (data not shown). In addition, *B. subtilis* is considered a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules (U.S. Department of Health and Human Services, 1986) and the European Federation of Biotechnology guidelines (Frommer *et al*., 1989). It is also classified as risk group 1 microorganism that never causes disease in human or adverse effect on environment. Including antimicrobial substances and diarrheal toxin from BS11 was non-detectable. A bulk of BS11 can also be prepared at industrial scale by fermentation without risk for workers after exposure. Recently, *Bacillus subtilis* has been authorized in the list of additives in feeding stuffs published by the European Union Commission (Council Directive 70/524/EEC, 2004). Therefore, it is possible to use BS11 as a safe probiotic for black tiger shrimp. However, regarding recognition as safe for human consumption, risk assessment of BS11 should be further confirmed in the future.

4. Conclusion

BS11 identified as *Bacillus subtilis,* which are generally habituated in soil and harmless to animals and humans. In combination with previous studies that showed BS11's ability to enhance growth, survival and immunity of black tiger shrimp after supplement to shrimp feed, it appears that BS11 should be a good possible probiotic candidate for black tiger shrimp *Penaeus monodon*.

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