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Identification and Characterization of Actinomycetes Antagonistic to Pathogenic *Vibrio* spp. Isolated from Shrimp Culture Pond Sediments in Thua Thien Hue–Viet Nam

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The shrimp diseases in Thua Thien Hue province have mainly accounted for vibriosis caused by *Vibrio* spp. The addition of substantial amounts of antibiotics is still the method of choice for control the proliferation of pathogenic *Vibrio* spp. However the abuse of antibiotic agents has led to the emergence of antibiotic–resistant bacteria which may result to resistance transfer to pathogenic bacteria and accumulation of antibiotics in shrimp products. Therefore, developing an alternative strategy to control infections is urgently needed. Following this goal, in the present study, indigenous actinomycetes were isolated from shrimp pond sediments in Thua Thien Hue and strain A1 was screened for their activity against two pathogenic strains *Vibrio* sp. V7 and V10 derived from diseased shrimps from culture ponds in Thua Thien Hue. Based on the cell morphology observed with a JEOL 5410 LV scanning electron microscope and 16S rRNA nucleotide sequence used to search the GenBank database with the BLAST system, strain A1 was belong to *Streptomyces* genus and identified as *Streptomyces* sp. A1. The 16S rRNA sequence of *Streptomyces* sp. A1 has been deposited in the DDBJ/EMBL/GenBank database with the accession number HM854225. Besides, *Streptomyces* sp. A1 also showed ability to produce siderophore to acquire iron and extracellular enzymes to decompose organic compounds such as starch, protein and cellulose. Otherwise, *Streptomyces* sp. A1 was non–pathogenic to shrimp. Therefore, *Streptomyces* sp. A1 can be considered as not only a candidate for control the shrimp diseases but also a potential probiont for shrimp culture.

Keywords: Actinomycetes, probiotic, shrimp pond sediment, vibriosis, *Vibrio* spp.

INTRODUCTION

Thua Thien Hue province (Viet Nam) with the largest Tam Giang–Cau Hai coastal lagoon system of Southeast Asia has many favorable conditions for development of shrimp culture. In fact, remarkable increase in shrimp culture helps to restructure the rural economy in a positive direction. However, in recent years, a rapid, large–scale and often unplanned increase in brackish water shrimp culture ponds have resulted in shrimp epidemic diseases. The shrimp diseases have mainly accounted for vibriosis caused by *Vibrio* spp. The addition of substantial amounts of antibiotics is still the method of choice for control the proliferation of pathogenic *Vibrio* spp. But the abuse of antibiotics has resulted in the development of resistant strains and antibiotic residues in shrimp products. The antibiotic resistance determinants that have emerged and/or evolved in the aquaculture environments have been shown to be transmitted by horizontal gene transfer to bacteria of the terrestrial environment, including animal and human pathogens. The presence of residual antibiotics in commercialized shrimp products constitutes another problem with respect to human health because this can lead to an alteration of the normal human gut microflora and can generate problems of allergy and toxicity (Cabello, 2006). Given the world–wide trade in aquaculture products, health problems related to antibiotic use in aquaculture are not limited to producing countries, but are also relevant to importing countries. Actually, there were many events of returning consignments to Viet Nam from Japan for not maintaining the prescribed standards on residual antibiotics of the products. Therefore, to make the shrimp aquaculture industry more sustainable, alternative strategies to control infections are urgently needed. One of the most successful alternative method has been reported to be the use of probiotics as biological control agents in shrimp aquaculture. As far as you concern, most probiotics proposed as biological control agents in aquaculture are lactic acid bacteria (*Lactobacillus*, *Carnobacterium* etc.), *Bacillus* strains, and *Pseudomonas* strains (Verschuere *et al*., 2000). However, most such probiotics in Viet Nam are either imports or domestic products with un–known sources, resulting in a threat of spreading pathogenic and genetically modified strains.

As potential probiotic strains in shrimp culture, actinomycetes have many following advantages: (1) the production of antimicrobial and antiviral agents (Austin, 1989; Oskay *et al*., 2004); (2) the degradation of complex biological polymers, such as starch and protein (Barcina *et al*., 1987), lignocellulose, hemicellulose, pectin, keratin, and chitin (Williams *et al*., 1984) which shows the potential to involve in mineralization and nutrient cycles in the culture ponds and in feed utilization and digestion once getting colonized into the host intestine; (3) the competition for nutrients, particularly iron in

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marine microbes (Kesarcodi *et al*., 2008); (4) the mostly non–pathogenic to the target animals in aquaculture (Yang *et al*., 2007); and (5) the formation heat– and desiccation– resistant spores and the retention of viability during preparation and storage. However, reports on probiotics consisting of actinomycetes are meager.

This article gives an account of the actinomycetes strain antagonistic to pathogenic *Vibrio* spp. isolated from shrimp pond sediments in Thua Thien Hue and also deals with its other probiotic characteristics and sensitivity toward antibiotics. These results may facilitate studies which offer the potential application of actinomycetes for improving the shrimp aquaculture in Thua Thien Hue.

MATERIALS AND METHODS

Sample Collection and Processing

Sediment samples were collected from shrimp culture ponds at 3 different sites viz. Lang Co–Phu Loc district (16°13'37"N, 108°02'40"E), Thuan An–Phu Vang (16°31'12"N, 107°38'38"E), and Quang An–Quang Dien district (16°34'08"N, 107°33'44"E), Thua Thien Hue, Viet Nam. Collections of the samples were made at 3 different stages of shrimp culture (I, II and III), corresponding to 5, 60 and 105th days of culture during 4 months from January to May 2010 at the sampling sites. Six sediment samples of 5 g each were collected from 2–5 cm below the surface of shrimp pond, so that a total of 30 g represented at each of sample collections. Sediment samples were placed in small pre–labeled sterile plastic bags which were tightly sealed and transported immediately to the laboratory where the sediment samples were dried for one week to obtain the so–called air–dry state then crushed in a mortar and run through a 2–mm mesh sieve and kept in closed plastic bottles for storage at 4 °C.

Sediment properties

The pH (H_2O) and pH (KCl) were measured by the glass electrode method using a Hach–C535 (Hach, USA). Total organic carbon (OC), nitrogen (N) and phosphorus (P_2O_5) were determined by Tiurin's method, Kjeldahl's method and colorimetric method, respectively.

Isolation, identification and characterization of pathogenic *Vibrio* spp.

Vibrio spp. were isolated from muscle and liver of moribund diseased tiger shrimps (*Penaeus monodon*) collected at culture ponds in Thua Thien Hue by spread plate method on Thiosulphate Citrate Bile Sucrose Agar (TCBS, HiMedia), at 35 °C for 24–48 hours and kept on Tryptone Soya Agar (TSA, Becton Dickinson) slants containing 1.5% NaCl.

The pathogenicity of *Vibrio* spp. to juvenile tiger shrimp (*Penaeus monodon*) was tested by injecting intramuscularly into healthy shrimps at the site between the third and fourth abdominal segments (Vera *et al*., 1992) and maintained in plastic basins containing sterile brackish water. Shrimp inoculated with sterile saline served as control. Mortalities were recorded up to 48 h post–inoculation.

Their phylogenetic characteristics were determined based on the nucleotide sequences of 16S rRNA. DNA extraction of *Vibrio* spp. was conducted following protocol of Sambrook and Russell (2001). PCR reactions were carried out in a final volume of 25 μl containing 0.5 μl of template; 2.5 μl buffer taq (10X); 3 μl $MgCl_2$ (25 mM); 0.625 μl of each dNTP (10 mM); 1.4 μl of each primer; and 0.3 μl of Taq DNA polymerase (5U/μl). The bacterial 16S rRNA targeted primer pair consisting of 341F and 907R. The amplifications were programmed for an initial denaturation of 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 55 sec at 58 °C and 1 min at 72 °C, and a final extension of 7 min at 72 °C. The 16S rRNA nucleotide sequences were compared with available 16S rRNA nucleotide sequences in GenBank using the BLAST system and submitted to DDBJ/EMBL/GenBank to get respective accession numbers.

The antibiotic susceptibility of *Vibrio* spp. was performed by disk diffusion method established by Bauer *et al*. (1966) and standardized by the National Committee for Clinical Laboratory Standards (NCCLS).

Isolation of actinomycetes

Isolation media consisted of the following (1) the starch casein agar (SCA) (soluble starch 10 g, casein 0.3 g, K_2HPO_4 2 g, KNO_3 2 g, NaCl 2 g, $MgSO_4$. $7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4$. $7H_2O$ 0.01 g, agar 15 g, distilled water to 1 L, pH 7.6) added with filtered (0.2–μm pore size) nystatin (25 μg/l) after sterilization at 45–50 °C to inhibit the growth of fungi and nalidixic acid (10 μg/l) to inhibit the growth of bacteria; (2) the AIM medium (soluble starch 20 g, KNO_3 1 g, NaCl 0.5 g, K_2HPO_4 0.5g, $MgSO_4$ 0.5 g, $FeSO_4$ 20 μM, agar 15 g, seawater to 1 L, pH 7.6) supplemented with nystatin (25 μg/l) and nalidixic (15 μg/l). One gram samples of dried sediments were diluted (10^{-2} to 10^{-5}) in sterile saline solution (0.85% w/v NaCl). 100 μl of each dilution was plated onto isolation media in triplicate petri dishes. The inoculated plates were incubated at 35° C for 7 days. After incubation, actinomycetes isolates distinguished from other microbial colonies by characteristics such as tough, leathery colonies which are partially submerged into the agar were purified by streak plate method and maintained on SCA slant at 4 °C.

Activities against *Vibrio* spp. of actinomycetes isolates

The activities against pathogenic *Vibrio* strains of actinomycetes isolates were determined using the double–layer agar method. The actinomycetes were inoculated on petri dishes containing 15 ml SCA and incubated at 35 °C for 3 days. Then TCBS agar medium (HiMedia) was poured onto the basal layer containing actinomycete colonies. *Vibrio* strains were inoculated in flask containing 50 ml peptone alkaline (10 g peptone, NaCl 10 g, distilled water to 1 L, pH 8.5) at 30 °C for 24 hours. Then *Vibrio* spp. was plated onto the top layer, respectively. The inhibition zones were measured after incubation at

35 °C for 24 hours (You *et al*., 2005). The actinomycetes strains with high activities against pathogenic *Vibrio* spp. were screened for further studies.

Identification of actinomyces

Electron microscopy

Cultures grown SCA medium were harvested, and fixed with 2.5% (w/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 30 min and transferred to a gold mesh for 1 min to fix on it. The samples were rinsed lightly with deionized water and then dehydrated sample with a series of ethanol concentrations (25, 50, 75 and 100%). The resulting preparations were transferred to T–butyl, dried with a lyophilizer, coated with gold, and observed with a JEOL 5410 LV scanning electron microscope. The morphology of actinomycetes was photographed.

Nucleic acid extraction, 16S rRNA amplification, sequencing and analysis

Colonies were transferred into 1.5 ml eppendorf tubes containing 200 µl 10 mM TE (pH 8.0) and vortex thoroughly. The suspensions were added with 200 µl 20% SDS, vortex gently, incubated at 65 °C for 4 min, and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatants and pellets were transferred into new eppendorf tubes separately. Eppendorf tubes containing cell pellets were placed into the microwave oven set at low level, for 30 sec with 3 times of repeats. These cell pellets were mixed well the supernatants. The suspensions were added with 500 µl chloroform/isoamyl alcohol (24:1), turned upside down and centrifuged at 12,000 rpm for 10 min at 4 °C. The aqueous (upper) phases were carefully transferred to new eppendorf tubes, precipitated with 400 µl isopropanol (1:1) supplemented with 10% sodium acetate, kept at –20 °C for 2–3 hours and centrifuged at 12,000rpm for 10 min at 4°C. The supernatants were discarded. The pellets were added with 1ml 70% ethanol, centrifuged at 12,000 rpm for 10 min at 4 °C and dried in a speed vacuum concentrator. The pellets were dissolved in 20 µl sterile deionnized water. The DNA obtained is further referred to as crude DNA. The 16S rRNA sequencing temple was amplified by PCR using primers 341F and 907R. Reaction mixture (25 µl) contained 0.5 µl of template; 2.5 µl buffer taq (10X); 3 µl $MgCl_2$ (25 mM); 0.625 µl of each dNTP (10 mM); 1.4 µl of each primer; and 0.3 µl of Taq DNA polymerase (5U/µl). All sequencing reactions were carried out with an ABI PRISM 3100 genetic analyzer programmed for an initial denaturation of 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 55 sec at 58 °C and 1 min at 72 °C, and a final extension of 7 min at 72 °C. The 16S rRNA sequences (540 bp) were used to search the GenBank database with the BLAST system to reveal closest matches to the 16S rRNA nucleotide sequences for known species and submitted to DDBJ/EMBL/GenBank to get respective accession number.

Production of extracellular enzymes

Ability to produce extracellular enzymes such as amylase, protease and cellulase of actinomycetes were tested on SCA plates containing separately 1% starch, 1% CMC (carboxymethyl cellulose), and 1% casein, at 35 °C for 3 days. Lugol's reagent was used to find the degradation of starch and CMC, whereas Fraziaer's reagent was used to find the degradation of casein.

Siderophore production

Production of siderophores by the actinomycetes isolate was determined using CAS assay solutions in cell–free culture supernatants (Payne, 1994). For the detection of siderophores by the CAS assay method, the isolate was grown in iron deficient defined medium MM9, 100 mM Pipes buffer, 0.2% glucose, 0.095% $MgCl_2$, 0.011% $CaCl_2$ at 30 °C for 72 hours. Cell free culture supernatants were obtained by centrifugation of the cultures at 6,000 rpm, 4 °C for 15 min, followed by filtration using 0.2 µm pore size filters. One milliliter of cell–free culture supernatant thus prepared and 1 ml of CAS assay solution were mixed, then added 20 µl shuttle solution (0.2 M 5–sulfosalicylic acid) and after equilibrium was reached, absorbance of this solution at 630 nm was recorded using spectrophotometer. The minimal medium was served as a blank and the minimal medium plus CAS assay solution plus shuttle were served as a reference (r). The sample (s) should have a lower reading than the reference. Siderophore units are defined as $[(A_r - A_s)/A_r]100$ = % siderophore units.

Pathogenicity of actinomycetes to *P. monodon* larvae

The larvae of *P. monodon* (stage PL 9) were maintained for 4 days in brackish water (20 ppt) and fed with commercial feed. 50 PL were distributed in 20–L plastic plastic basins containing 10 L of sterile brackish water. The actinomycetes was cultured in SCA at 35 °C for 3 days and were scraped from the surface of the plates using a sterile loop and suspended in sterile saline solution to saline solution to McFarland 0.5 standard, corresponding to approximate 10^8 cfu per milliliter. The shrimp larvae were challenged at 10^5 cfu per milliliter. The experiment was carried out in triplicate. The larval mortality was monitored for up to 4 days.

Antibiotic susceptibility of actinomycetes

The antibiotic susceptibility was performed by disk diffusion method established by Bauer *et al*. (1966) and standardized by National Committee for Clinical Laboratory Standards (NCCLS). A total of 7 antibiotic discs (bioMérieux, France) which includes ampicillin (10 µg), erythromycin (15 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), tetracycline (30 µg), gentamicin (10µg), penicillin–G (10 µg) were employed. The actinomycetes suspension with the same density as the McFarland 0.5, was streaked with a sterile swab over the entire surface of Muller– Hinton agar plates and the antimicrobial discs were soon applied to the plates. The plates were incubated at 35 °C for 24 hours. Inhibitory zone size was measured in millimeter and compared with the standard interpretative chart of *Vibrio cholerae* (except ciprofloxacin, erythromycin and penicillin–G of

Enterobacteriaceae, Enterococcus and staphylococci, respectively) to determine their antibiotic sensitivity.

RESULTS

Sediment properties

Sediment samples were collected from three different locations and their properties were determined (Table 1). pH (H_2O) averaged 7.6 in a range of 7.1 and 8.0. It is likely to suitable because shrimp pond bottom soils always need to have a pH of 7.5 or above to encourage decomposition of organic matter. The high pH reflects the effect of the applications of liming materials into dry shrimp pond bottom.

The highest contents of organic carbon (OC), nitrogen (N) and P_2O_5 were found in the Lang Co–Phu Loc (I), Thuan An–Phu Vang (Ⅱ) and Quang An–Quang Dien (Ⅲ) sediments, respectively. The Quang An–Quang Dien sample was likely to be the one with highest content of the organic matter. The content of organic carbon averaged 1.07% with a range of 0.68–2.06%. These findings also agree with the observation that aquaculture ponds situated on mineral soil seldom contain more than 3% organic carbon (Boyd, 1995). A factor of 1.9 times organic carbon concentration is thought to provide a reasonable estimate of organic matter in surface soil (Nelson and Sommers, 1982). Nitrogen averaged 0.19% with a range of 0.11–0.39% and P_2O_5 averaged 0.55% with a range of 0.03–0.09%.

Isolation, identification and characterization of pathogenic *Vibrio* spp.

Based on typical colonial morphology of *Vibrio* spp. on TCBS agar after incubating at 35 ˚C, for 24–48 hours, 15 strains were isolated from diseased shrimp samples (Fig. 1) and coded from V1 to V15. Then they were selected for the pathogenicity to juvenile tiger shrimp (*Penaeus monodon*). The severity of pathogenicity depends on strain of *Vibrio* involved. Strains V7 and V10 were found to cause 100% mortality of shrimp at the infection levels of 10^6 and 10^7 cfu/g, respectively. No mortality was observed for shrimps in the control group and remains. Therefore, strains V7 and V10 were used for further studies.

The 16S rRNA nucleotide sequence of strain V7 (Fig. 2) reached a 98.5% indentity with 16S rRNA nucleotide sequence of *Vibrio harveyi* ACMM 642; *V. campbellii* ATCC 25920^T; *V. parahaemolyticus* RIMD 2210633; *V. rotiferianus* R–14939^T; *V. owensii* 47666–1. Meanwhile, the 16S rRNA nucleotide sequence of strain V10 (Fig. 3) showed an identity of 99.6% with *Vibrio parahaemolyticus* ATCC 17802; *V. parahaemolyticus* JGX080708; *V. alginolyticus* GCSL 29; *Vibrio* sp. V170; and of 98.5% with *V. harveyi* SW–4; *Vibrio* sp. LC1–257. The strains V7 and V10 could be identified as *Vibrio* sp. V7 and *Vibrio* sp. V10, respectively. The 16S rRNA nucleotide sequences of strains V7 and V10 determined in this study have been deposited in the DDBJ/EMBL/GenBank database with the accession numbers HM854227

Table 1. Properties of sediments from shrimp ponds in Thua Thien Hue province

Sample	Stage	pH (H_2O)	pH (KCl)	OC (%)	N (%)	P_2O_5 (%)
Lang Co–Phu Loc	I	8.0	6.5	2.06	0.15	0.08
	Ⅱ	7.9	7.7	0.74	0.15	0.07
	Ⅲ	7.7	7.6	0.68	0.16	0.05
Thuan An–Phu Vang	I	7.9	7.6	0.86	0.18	0.04
	Ⅱ	7.1	6.7	0.86	0.11	0.03
	Ⅲ	7.4	7.0	0.80	0.18	0.03
Quang An–Quang Dien	I	7.9	7.6	1.22	0.24	0.08
	Ⅱ	7.2	6.6	1.13	0.39	0.08
	Ⅲ	7.2	6.8	1.28	0.17	0.09

Stage I; 5th day, Stage Ⅱ; 60th day, Stage Ⅲ; 105th day of shrimp culture, OC; organic carbon

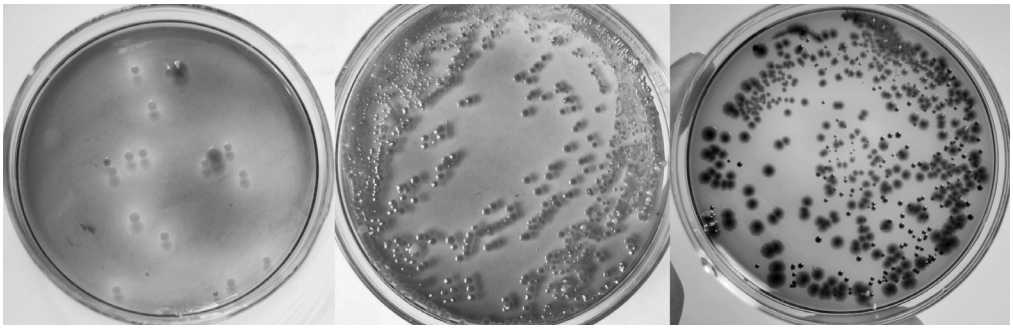

Fig. 1. Colonial morphology of *Vibrio* spp. isolated from (a) Lang Co– Phu Loc, (b) Quang An– Quang Dien, (c) Loc Dien– Phu Loc on TCBS at 35 ˚C, 24–48 h.

Table 2. Zone diameter interpretive standards and antibiotic susceptibility of *Vibrio* sp. V7 and *Vibrio* sp. V10

Antimicrobial agent (Disk Content)	Diameter of zone of inhibition (mm)			*E. coli* ATCC 25922	*S. aureus* ATCC 25923	*Vibrio* sp. V7		*Vibrio* sp. V10	
	Resistant (R)	Intermediate (I)	Susceptible (S)						
Ampicillin (10 μg)	≤ 13	14 – 16	≥ 17	23	35	15	I	7	R
Erythromycin (15 μg)	≤ 13	14 – 22	≥ 23	10	30	21	I	20	I
Chloramphenicol (30 μg)	≤ 12	13 – 17	≥ 18	26	26	34	S	35	S
Ciprofloxacin (5 μg)	≤ 15	16 – 20	≥ 21	40	28	35	S	30	S
Tetracycline (30 μg)	≤ 14	15 – 18	≥ 19	25	28	27	S	29	S
Gentamicin (10 μg)	≤ 12	13 – 14	≥ 15	20	22	14	I	19	S
Penicillin– G (10 μg)	≤ 28	not suitable	≥ 29	7	37	7	R	7	R

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   1 CTTCGGGTTG TAAAGCACTT TCAGTCGTGA GGAAGGTAGT GTAGTTAATA GCTGCATTAT
  61 TTGACGTTAG CGACAGAAGA AGCACCGGCT AACTCCGTGC CAGCAGCCGC GGTAATACGG
 121 AGGGTGCGAG CGTTAATCGG AATTACTGGG CGTAAAGCGC ATGCAGGTGG TTTGTTAAGT
 181 CAGATGTGAA AGCCCGGGGC TCAACCTCGG AATTGCATTT GAAACTGGCG AGACTAGAGT
 241 ACTGTAGAGG GGGGTAGAAT TTCAGGTGTA GCGGTGAAAT GCGTAGAGAT CTGAAGGAAT
 301 ACCGGTGGCG AAGGCGGCCC CCTGGACAGA TACTGACACT CAGATGCGAA AGCGTGGGGA
 361 GCAAACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC GATGTCTACT TGGAGGTTGT
 421 GGCCTTGAGC CGTGGCTTTC GGAGCTAACG CGTTAAGTAG ACCGCCTGGG GAGTACGGTC
481 GCAAGATTAA A
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Fig. 2. The 16S rRNA nucleotide sequence of strain V7.

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   1 GGGCGCAAGC CTGATGCAGC CATGCCGCGT GTGTGAAGAA GGCCTTCGGG TTGTAAAGCA
  61 CTTTCAGTCG TGAGGAAGGT GGTAGTGTTA ATAGCACTAT CATTTGACGT TAGCGACAGA
 121 AGAAGCACCG GCTAACTCCG TGCCAGCAGC CGCGGTAATA CGGAGGGTGC GAGCGTTAAT
 181 CGGAATTACT GGGCGTAAAG CGCATGCAGG TGGTTTGTTA AGTCAGATGT GAAAGCCCGG
 241 GGCTCAACCT CGGAATTGCA TTTGAAACTG GCAGACTAGA GTACTGTAGA GGGGGGTAGA
 301 ATTTCAGGTG TAGCGGTGAA ATGCGTAGAG ATCTGAAGGA ATACCGGTGG CGAAGGCGGC
 361 CCCCTGGACA GATACTGACA CTCAGATGCG AAAGCGTGGG GAGCAAACAG GATTAGATAC
 421 CCTGGTAGTC CACGCCGTAA ACGATGTCTA CTTGGAGGTT GTGGCCTTGA GCCGTGGCTT
 481 TCGGAGCTAA CGCGTTAAGT AGACCGCCTG GGGAGTACGG TCGCAAGATT AAAACTCAAA
541 TGAATTGACG GGAA
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Fig. 3. The 16S rRNA nucleotide sequence of strain V10.

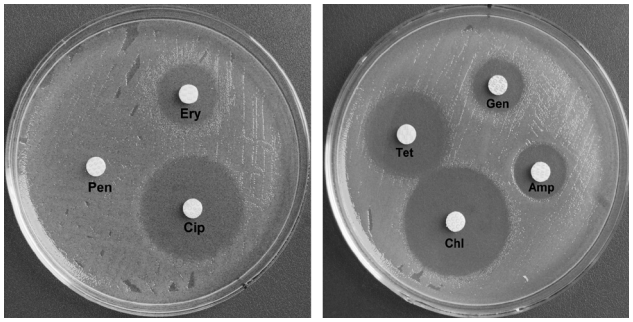

Fig. 4. Antibiotics sensitivities of *Vibrio* sp. V7.

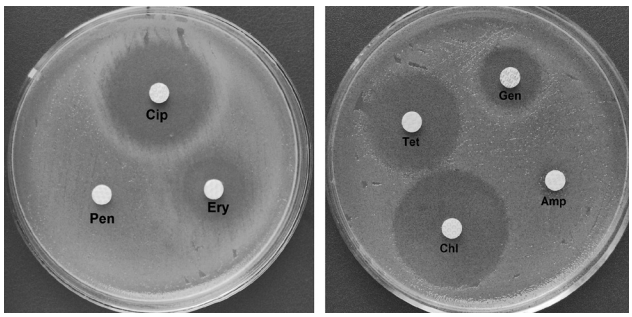

Fig. 5. Antibiotics sensitivities of *Vibrio* sp. V10.

and HM854228, respectively.

Antibiotics sensitivities tests revealed *Vibrio* sp. V7 and *Vibrio* sp. V10 to be sensitive to chloramphenicol (Chl), ciprofloxacin (Cip), tetracycline (Tet), intermediate to erythromycin (Ery), and resistant to penicillin–G (Pen). However, *Vibrio* sp. V10 was resistant to ampicillin (Amp) and sensitive to gentamicin (Gen), while *Vibrio* sp. V7 was intermediate to ampicillin (Amp) and gentamicin (Gen) (Table 2, Fig. 4, 5).

Isolation of actinomycetes

A total of 50 strains of actinomycetes was isolated using three different agar media from the sediment samples collected at different locations. Strain A1 showed good growth on SCA after 3 days (Fig. 6).

Fig. 6. Colonial morphology and biomass of strain A1 isolated from Quang An–Quang Dien sediment on SCA medium.

Activities against *Vibrio* spp. of actinomycetes isolates

All actinomycetes isolates were screened for their activities against two strains *Vibrio* sp. V7 and V10 using the double–layer agar method. Of these, strain A1 showed the highest activity against both strains V7 and V10 (Fig. 7). The diameters of zone antagonistic to strains V7 and V10 were 40 and 36 mm, respectively. Therefore, strain A1 was selected for further studies.

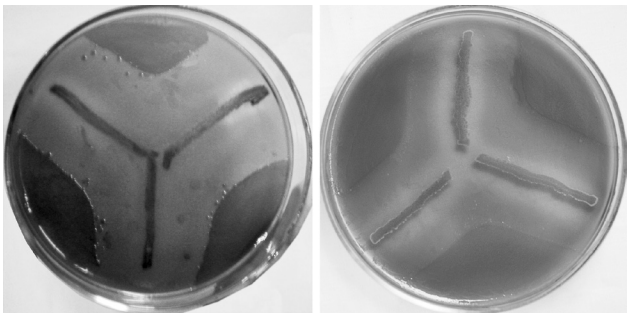

Fig. 7. Activities against *Vibrio* sp. V7 (left) and V10 (right) of strain A1.

Identification of strain A1

A photograph of strain A1 taken using a JEOL 5410 LV scanning electron microscope was shown in Fig.8. The 16S rRNA nucleotide sequence of strain A1 was determined and presented in Fig. 9. The result of the homology search with GeneBank database using the BLAST system indicated that the 16S rRNA nucleotide sequence of strain A1 had a 95.5% identity with that of *Streptomyces* sp. An53; *S. griseoaurantiacus* DSM 40430; *Streptomyces* sp. ME02–6978.2a; *S. griseoaurantiacus* NBRC 15440^{T}. Therefore, strain A1 was considered to belong genus *Streptomyces* and identified as *Streptomyces* sp. A1. The 16S rRNA nucleotide sequence of *Streptomyces* sp. A1 has been deposited in the DDBJ/EMBL/GenBank database with the accession number HM854225.

Production of extracellular enzymes

Ability to produce extracellular enzymes to decompose organic compounds, such as starch, protein and cellulose, by strain A1 also was presented in Fig. 10. It may be due to actinomycetes are primarily saprophytic soil–dwelling organisms (Williams *et al*., 1984). This help to degrade the unconsumed feed and feces in the culture pond, in addition to the possible role of these enzymes in the nutrition of the shrimps by improving feed digestibility and feed utilization. Once actinomycetes get colonized into the host intestine, the exoenzymes produced by actinomycetes may be helpful in facilitating feed utilization and digestion (Das *et al*., 2008).

Siderophore production

Streptomyces sp. A1 was found to produce 40% siderophore units as detected by the CAS assay technique. Therefore, *Streptomyce*s sp. A1 could not only inhibit the growth of all *Vibrio* spp., but also produce siderophores.

Pathogenicity of *Streptomyces* sp. A1 to *P. monodon* larvae

The pathogenicity of *Streptomyces* sp. A1 test on larvae of *P. monodon* indicated that the strain A1 did not cause any significant mortality after 4 days of challenge (10^5 cfu per milliliter). This result agree with the virulence factor database (Yang *et al*., 2007) (accessed on 14 September 2008) suggested that *Streptomyces* will not cause harm to the target animals in aquaculture.

Antibiotic susceptibility of *Streptomyces* sp. A1

Antibiotics sensitivities tests revealed *Streptomyces* sp. A1 to be sensitive to chloramphenicol (Chl), ciprofloxacin (Cip) and gentamicin (Gen), to intermediate to erythromycin (Ery), and resistant to ampicillin (Amp), tetracycline (Tet) and penicillin–G (Pen) (Table 3, Fig. 11).

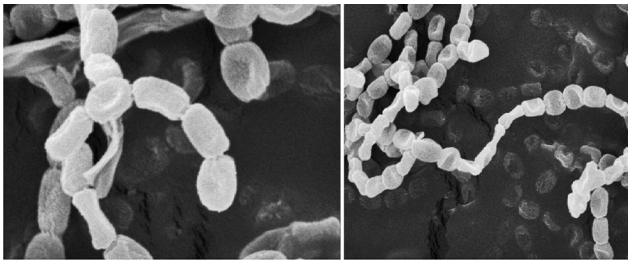

Fig. 8. Microscopic characteristic of *Streptomyces* sp. A1.

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   1 ATGGGCGAAA GCCTGATGCA GCGACGCCGC GTGAGGGATG ACGGCCTTCG GGTTGTAAAC
  61 CTCTTTCAGC AGGGAAGAAG CGAAAGTGAC GGTACCTGCA GAAGAAGCGC CGGCTAACTA
 121 CGTGCCAGCA GCCGCGGTAA TACGTAGGGC GCAAGCGTTG TCCGGAATTA TTGGGCGTAA
 181 AGAGCTCGTA GGCGGCTTGT CACGTCGGTT GTGAAAGCCC GGGGCTTAAC CCCGGGTCTG
 241 CAGTCGATAC GGGCAGGCTA GAGTTCGGTA GGGGAGATCG GAATTCCTGG TGTAGCGGTG
 301 AAATGCGCAG ATATCAGGAG GAACACCGGT GGCGAAGGCG GATCTCTGGG CCGATACTGA
 361 CGCTGAGGAG CGAAAGCGTG GGGAGCGAAC AGGATTAGAT ACCCTGGTAG TCCACGCCGT
 421 AAACGGTGGG CACTAGGTGT GGGCAACATT CCACGTTGTC CGTGCCGCAG CTAACGCATT
 481 AAGTGCCCCG CCTGGGGAGT ACGGCCGCAA GGGCTAAACT AGGGGGAAAT GTGACGGA
```

Fig. 9. The 16S rRNA nucleotide sequence of strain *Streptomyces* sp. A1.

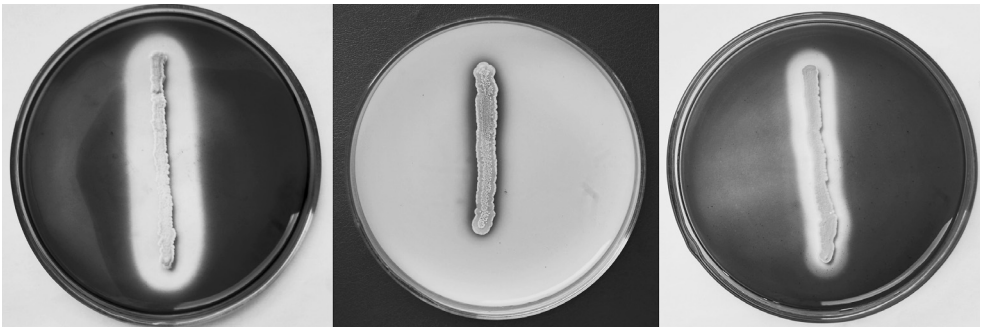

Fig. 10. Degradation of starch (left), casein (middle), cellulose (right) of *Streptomyces* sp. A1.

Table 3. Zone diameter interpretive standards and antibiotic susceptibility of *Streptomyces* sp. A1

Antimicrobial agent (Disk Content)	Diameter of zone of inhibition (mm)			*E. coli* ATCC 25922	*S. aureus* ATCC 25923	*Streptomyces* sp. A1	
	Resistant (R)	Intermediate (I)	Susceptible (S)				
Ampicillin (10 μg)	≤ 13	14 – 16	≥ 17	23	35	0	R
Erythromycin (15 μg)	≤ 13	14 – 22	≥ 23	10	30	20.5	I
Chloramphenicol (30 μg)	≤ 12	13 – 17	≥ 18	26	26	24.0	S
Ciprofloxacin (5 μg)	≤ 15	16 – 20	≥ 21	40	28	29.0	S
Tetracycline (30 μg)	≤ 14	15 – 18	≥ 19	25	28	13.0	R
Gentamicin (10 μg)	≤ 12	13 – 14	≥ 15	20	22	34.5	S
Penicillin– G (10 μg)	≤ 28	not suitable	≥ 29	7	37	0	R

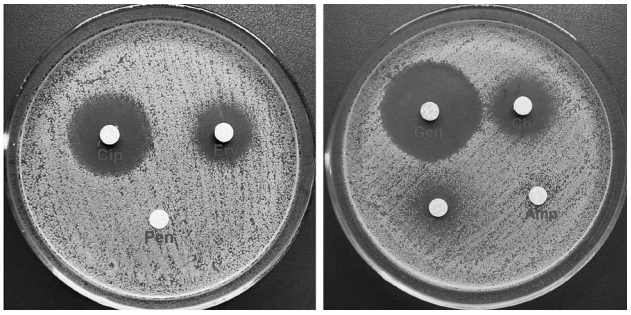

Fig. 11. Antibiotics sensitivities of *Streptomyces* sp. A1.

DISCUSSION

Many factors affect the concentration of organic matter in pond soils. Soil matter used in pond construction may vary in organic matter concentration among sites, and methods of pond management will affect rates of both input and decomposition of organic matter in pond soils. Within a given pond, there is usually an increase in soil organic matter in the surface 5 cm layer during a growing season (Gately, 1990). As far as you concern, organic matter with a C/N ratio of 10 to 15 will decompose much faster than organic matter with higher C/N ratios (Boyd, 1992). In this study, lower C/N ratio suggested the presence of more easily degradable organic matter in the shrimp pond sediments of Thua Thien Hue, particularly in the sediment of Quang An–Quang Dien. It is suitable that *Streptomyces* sp. A1 antagonistic to pathogenic strains *Vibrio* sp. V7 and V10 was derived from the sediment of Quang An–Quang Dien. The mortality (100%) of shrimps intramuscularly injected with *Vibrio* sp. V7 and V10 was observed after only 8 and 5 hours of injection, respectively. This phenomenon should be study further. Besides, *Vibrio* sp. V7 was resistant to penicillin–G and *Vibrio* sp. V10 was resistant to penicillin–G and ampicillin. This result was similar to that of Najiah *et al*. (2008) reported that 90% of *V. harveyi* isolates were mostly resistant to ampicillin. Besides, the resistance to ampicillin in *V. harveyi* isolated from aquaculture facilities in Asia and South America was reported (Teo *et al*., 2002; Molina–Aja *et al*., 2002; Nakayama *et al*., 2006). Also, *V. parahaemolyticus* isolated from red disease affected shrimp was resistant to ampicillin and penicillin–G (Jayasree *et al*., 2006). The development of resistance was accounted for use of antibiotics in shrimp culture systems. The alternative measures that have recently been developed to control disease caused by *V. harveyi* and closely related bacteria include phage therapy, the use of short–chain fatty acids and polyhydroxyalkanoates, quorum–sensing disruption, probiotics and green water. Of them, the application of probiotics have a longer history (Tom *et al*., 2007). Possible modes of action for probiotics include (i) production of inhibitory compounds, (ii) competition for nutrients, (iii) competition for adhesion sites in the gastrointestinal tract, (iv) enhancement of theimmune response and (v) production of essential nutrients such as vitamins and fatty acids, and enzymatic contribution to digestion (Verschuere *et al*., 2000; Vine *et al*., 2006). Given this circumstance, actinomycetes should be considered as potential probiotic strains in shrimp culture. You *et al*. (2005) described the potential of actinomycetes against shrimp pathogenic *Vibrio* spp. Kumar *et al*. (2006) extracted the antibiotic product from marine actinomycetes and incorporated it into feed to observe the *in vivo* effect on white spot syndrome virus in black tiger shrimp. Das *et al*. (2006) reported a preliminary study on the effect of probiotic supplementation of *Streptomyces* on the growth and aids in preventing disease of black tiger shrimp, *Penaeus monodon* (Fabricius). You *et al*. (2007) recommended the use of actinomycetes to prevent the disease caused by *Vibrio* spp. Folowing this trend, *Streptomyces* sp. A1 isolated in the present study showed the activity against two pathogenic strains *Vibrio* sp. V7 and V10 through production of inhibitory compounds and siderophore. The likely mode of action against pathogenic bacteria of *Streptomyces* projected that it release antibiotics in a sort of biochemical warfare to eliminate the competing microorganisms from the environment. These antibiotics are small molecules and interfere with gyrase protein, which assists in DNA replication. As a result, pathogenic bacteria are not able to divide normally. However, *Streptomyces* protects itself from its own antibiotics by the production of efflux pumps (used against the influx of antibiotics), ribosomal protection proteins (protect ribosome and prevents interfering with

protein synthesis), and modifying enzymes (neutralize antibiotics by the production of acetyl or phosphate groups) (Das *et al*., 2008). Iron is a limiting bioactive metal in seawater and essential for the growth of marine bacteria. Thus, marine bacteria have to develop some strategies to acquire iron. The major strategy is the production and utilization of siderophores. Siderophores, low molecular weight high–affinity ferric iron chelators, are synthesized and secreted by many microorganisms in response to iron deprivation. The marine actinomycetes that can produce siderophores could adapt to the iron stress conditions in marine environment, even compete for iron with the pathogens. Competition for iron is also a possible mechanism for aquaculture probiotics to control the pathogens. So the siderophore–producing *Streptomyces* strains could influence the growth of pathogenic *Vibrio* spp. by competition for iron in marine sediments. Otherwise, *Streptomyces* can form heat– and desiccation–resistant spores, and the probiotic products would be stable in preservation. *Streptomyces* sp. A1 can be mass–cultured, harvested, and fortified to feed. In this case, the positive effect may be obtained (Kumar *et al*., 2006). Therefore, in the present study, *Streptomyces* sp. A1 will be promising candidates as aquaculture probiotics.

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