Characterization of Actinomycetes Antagonistic to Vibrio parahaemoliticus Isolated from Shrimp Pond Sediment

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Abstract: A new emerged lethal disease that termed EMS (Early Mortality Syndrome) or AHPNS (Acute Hepatopancreatic Necrosis Syndrome) caused by *Vibrio parahaemolyticus* had been added to list of shrimp diseases during last recent years. However, there are no currently available methods to treat EMS. Given this circumstance, developing an alternative strategy to control infections, especially in countries found that antibiotics are not effective against EMS as Vietnam, is urgent need. In this study, a *Streptomyces* sp. A8 strain isolated from shrimp pond sediments in Thừa Thiên Huế showed the high activity against *V. parahaemolyticus* V6 and production of extracellular enzymes to decompose organic compounds which reveals the potential to involve in mineralization and nutrient cycles in the shrimp culture ponds. The *Streptomyces* sp. A8 strain was only resistant to several common antibiotics as ampicillin, tetracycline and penicillin-G. Selected cultivative conditions for biomass production and antagonistic activity to *V. parahaemolyticus* V6 of *Streptomyces* sp. A8 were 96 hours, pH 8.0, 35°C in SCB medium with concentrations of starch, casein, NaCl, DL- α -alanine and vitamin B6 were 13%, 0.6%, 16%, 0.6% and 0.02%, respectively. When being selected fermented, a large amount of *Streptomyces* sp. A8 biomass (15.0 g/L) was harvested.

Keywords: Actinomycetes, Streptomyces sp. A8, shrimp ponds, Vibrio parahaemolyticus, early mortality syndrome.

1. Introduction

EMS or more technically known as AHPNS should be considered as a new emerging shrimp disease that has been attacked to shrimp farms in Southeast Asia. It named as EMS due to mass mortality during few days after shrimp post larvae stoking. EMS has spread to Vietnam in 2010. This disease decreased the mass production from 70,000 tons in 2010 to 40,000 and 30,000 tons in 2011 and 2012, respectively. The economic lost was estimated 570,000 till 7,200,000 USD on 2011 and 2012 [1]. Despite of trying to disease control during last recent years, it is not under control and made severe mortality in 2014. Recently, the scientists found that EMS/AHPNS could be initiated by a bacterial agent that termed *V. parahaemolyticus* is transferred through oral and then localizes the shrimp gastrointestinal tract and create a poison that causes tissue devastation and invalidism of the shrimp digestive system known as the hepatopancreas [1, 2]. Besides the diagnostic

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tests for rapid detection of EMS that will enable improved management of ponds and help lead to a long-term solutions for the disease, there are no currently available methods to treat EMS. Importantly, it has been found in countries that antibiotics are not effective against EMS. Sensitivity tests have shown the bacteria have already developed resistance to full range of antibiotics [3]. Whereas, as potential biocontrol agents in shrimp aquaculture, actinomycetes have many following advantages (i) the production of antimicrobial and antiviral agents [4]; (ii) the degradation of complex organic compounds [5]; (iii) the competition for nutrients [6]; (iv) the mostly non-pathogenic to the target animals in aquaculture [7]; and (v) the formation heat- and desiccation- resistant spores and the retention of viability during preparation and storage. However, reports on the use of actinomycetes preparations for sustainable shrimp aquaculture are meager. This article gives an account of characterization of actinomycetes antagonistic to V. parahaemoliticus as the causative agent of EMS isolated from shrimp pond sediments in Thừa Thiên Huế.

2. Materials and methods

2.1. Isolation and identification of Vibrio parahaemolyticus

V. parahaemolyticus was isolated from moribund diseased shrimps (Litopenaeus vannamei) 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 isolate was identified based on morphological, biochemical phylogenetic characteristics. and The morphological and biochemical characteristics were determined as given in Cowan and Steel's manual [8] and Bergey's Manual of Systematic phylogenetic Bacteriology [9]. The characteristics was determined based on 16S rRNA nucleotide sequence. The bacterial DNA extraction was conducted following protocol of Sambrook and Russell (2001) [10]. PCR reaction was carried out in a final volume of 25 ul containing 0.5 ul of template; 2.5 ul buffer taq (10X); 3 µl MgCl₂ (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 16S rRNA targeted primer pair consisting of 341F and 907R. The amplification was 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 sequence was compared with available 16S rRNA nucleotide sequences in GenBank using the BLAST.

2.2. Isolation of actinomycetes

The starch casein agar (SCA) (soluble starch 10 g, casein 0.3 g, K2HPO4 2 g, KNO3 2 g, NaCl 2 g, MgSO4. 7H2O 0.05 g, CaCO3 0.02 g, FeSO4. 7H2O 0.01 g, agar 15 g, distilled water to 1 L, pH 7.6) added with filtered (0.2 μ m pore size) nystatin (25 μ g/l) after sterilization at 45-50°C to inhibit the growth of fungi and nalidixic acid (10 μ g/l) to inhibit the growth of bacteria supplemented with nystatin (25 μ g/l) and nalidixic (15 μ g/l) was used for actinomycetes isolation. One gram samples of dried sediments were diluted (10-2 to 10-5) in sterile saline solution (0.85% w/v NaCl). 100 μ l of each dilution was plated onto isolation medium 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.

2.3. Activity against V. parahaemolyticus strain

The activities against *V. parahaemolyticus* strain of actinomycetes isolates were determined using the double-layer agar method [11]. The actinomycetes were inoculated on

petri dishes containing 15 ml SCA and incubated at 35°C for 3 days. Then TCBS agar was poured onto the basal layer containing actinomycete colonies. *V. parahaemolyticus* strain was 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. After that, it was plated onto the top layer, respectively. The inhibition zones were measured after incubation at 35°C for 24 hours. The actinomycetes strain with highest activities against *V. parahaemolyticus* strain was selected for further studies.

Culture of such strain grown SCA medium was 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 1min to fix on it. The sample was rinsed lightly with deionized water and then dehydrated sample with a series of ethanol concentrations (25, 50, 75 and 100%). The resulting preparation was transferred to Tbutyl, dried with a lyophilizer, coated with gold, and observed with a JEOL 5410 LV scanning electron microscope. The morphology of actinomycetes was photographed. Besides, the phylogenetic characteristics was determined based on 16S rRNA nucleotide sequence by the above- mentioned method.

2.4. Ability to degrade organic compounds of actinomycetes

Production of extracellular enzymes to degrade organic coumpounds such as amylase, protease and cellulase of actinomycetes was tested by the agar well-diffusion method 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.

2.5. 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 (bioMerieux, France) which includes ampicillin $(10\mu g)$, erythromycin $(15\mu g)$, chloramphenicol $(30\mu g)$, ciprofloxacin $(5\mu g)$, tetracycline $(30\mu g)$, gentamicin (10 μ g), penicillin-G (10 μ g) 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 charts of Vibrio cholerae (except ciprofloxacin, erythromycin and penicillin-G of Enterobacteriaceae, **Enterococcus** and staphylococci, respectively) to determine the antibiotic sensitivity.

2.6. Selection of some cultivative conditions and medium components for biomass production and antagonistic activity of actinomycetes to V. parahaemolyticus strain

The selected cultivatived conditions for biomass production and antagonistic activity of actinomyces was assessed by growing in SCB medium (above-mentioned SCA without agar) at (i) various pH (6.0, 7.0, 8.0 and 9.0); (ii) temperatures (25, 30, 35 and 40°C); and (iii) culture time (12, 24, 36, 48, 72, 96 and 120 hours).

The selection of nutrients in a pattern onefor biomass production at-a-time and antagonistic activity of actinomycetes to V. parahaemolyticus was assessed by growing in the SCB medium. Different C-sources (glucose, sucrose, maltose, lactose and starch) were screened as sole C-source at concentrations of 8-13 g/l in mineral fraction of SCB medium (g/l). Ammonium chloride, ammonium nitrate, ammonium sulphate, casein and urea were screened as sole N-source at concentrations of 0.1-0.6 g/l in the same medium with selected Csource. Sodium chloride was screened at

concentrations of 0-34 g/l. Some kinds of amino acids (DL- α -alanine, DL-nor-leucine, L-histidine, L-lysine and casamino acid) at concentrations of 0-0.2 g/l and vitamins (vitamin A, thiamine, pantothenic acid, pyridoxine and ascorbic acid) at concentrations of 0-0.02 g/l were screened as growth factors. All flasks were incubated on a shaker (New Brunswick, Innova 44R, Eppendorf, Germany) at selected conditions.

The biomass production was assessed via the constant weight of biomass harvested and antagonistic activity was determined by the agar well-diffusion method with 70 μ l of culture supernatant was pipetted into each well.

2.7. Fermentation for actinomycetes biomass production.

Actinomycetes was cultivated in selected culture medium sterilized for 15 min at 121° C in flasks on the shaker (New Brunswick, Innova 44R, Eppendorf, Germany) at 150 rpm, 35°C for 2 days. After that, this suspension was inoculated into the sterilized such fresh medium in a 10-L fermenter (Bioflo 610, Eppendorf, Germany) and then 120-L fermenter (Bioflo, Eppendorf, Germany) with a ratio of 1: 10 (v/v) and fermented at well-controlled selected conditions. After fermentation process, the fementor was left to stand for 30 min to allow the vegetative biomass (micro-colonies) of the actinomyces to settle. The biomass was harvested using sterile filter papers and a vacuum filter, washed with sterile distilled water at least three times, dried at 4-10°C until reached a constant weight.

3. Results and Discussion

3.1. Isolation, identification and characterization of V. parahaemolyticus

Based on typical colonial morphology of *V. parahaemolyticus* on TCBS agar after incubating at 35°C, for 24–48 hours, *Vibrio* spp. strains was isolated from diseased shrimp samples (Fig. 1).



Fig. 1. Colonial morphology of *Vibrio* spp. isolated on TCBS at 35°C for 24-48 h.

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	ААААСТСААА
541	TGAATTGACG	GGAA				

Fig. 2. The 16S rRNA nucleotide sequence of V. parahaemolyticus V6 strain.

The 16S rRNA nucleotide sequence of strain *Vibrio* sp. V6 (Fig. 2) reached a highest identity of 99.6% with *Vibrio parahaemolyticus* ATCC 17802; *V. parahaemolyticus* JGX080708. Besides, based on the morphological and biochemical characteristics corresponded to the references [8, 9], the strain V6 could be referred here as *Vibrio parahaemolyticus* V6.

3.2. Isolation of actinomycetes antagonistic to V. parahaemolyticus V6

A total of 10 strains of actinomycetes was isolated on SCA medium from the shrimp pond sediment samples and then screened for their activities against *V. parahaemolyticus* V6 strain using the double-layer agar method. Of these, A8 strain (Fig. 3) showed the highest activity against *V. parahaemolyticus* V6 strain with the diameter of antagonistic zone was 30 mm (Fig. 4). Therefore, A8 strain was selected for further studies. The likely mode of action against pathogenic bacteria of actinomycetes suggested that it release antibiotics in a sort of biochemical warfare to eliminate the competing microorganisms from the living 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. Whereas, actinomycetes 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 withprotein synthesis), and modifying enzymes (neutralize antibiotics by the production of acetyl or phosphate groups) [12]. The 16S rRNA nucleotide sequence of A8 strain was determined (Fig. 5). The result of the homology search with GeneBank database using the BLAST system showed that the 16S rRNA nucleotide sequence of A8 strain had a highest identity of 95.5% with that of Streptomyces sp. An 53. Besides, a photograph of strain A8 taken using a JEOL 5410 LV scanning electron microscope was shown in Fig.3. Therefore, the A8 strain could belong to Streptomyces genus and be referred here as *Streptomyces* sp. A8.



Fig. 3. Colonial and cell morphology of A8 strain isolated on SCA at 35°C for 7 days.



Fig. 4. Activity against V. parahaemolyticus V6 strain of A8 strain.

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ATGGGCGAAA GCCTGATGCA GCGACGCCG GTGAGGGATG ACGCCTTCG GGTTGTAAAC
CTCTTTCAGC AGGGAAGAAG CGAAAGTGAC GGTACCTGCA GAAGAAGCGC CGGCTAACT
CGTGCCAGCA GCCGCGGTAA TACGTAGGGC GCAAGCGTTG TCCGGAATTA TTGGGCGTAA
AGAGCTCGTA GGCGGCTTGT CACGTCGGTT GTGAAAGCCC GGGGCTTAAC CCCGGGTCTG
CAGTCGATAC GGCCAGGCTA GAGTTCGGTA GGGGAAGACG GAATCCTGG TGTAGCGGTG
AAATGCGCAG ATATCAGGAG GAACACCGGT GGCGAAGGCG GATCTCTGGG CCGATACTGA
CGCTGAGGAG CGAAAGCGTG GGGAACACT CCACGTTGTC CGTGCCGCAG CTAACGCCT
AAACGGTGGG CACTAGGTGT ACGCCGCAA GGCTAAACT AGGGGAAAT GTGACGAT
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Fig. 5. The 16S rRNA nucleotide sequence of Streptomyces sp. A8 strain.

On the other hand, the antimicrobial agent from the culture broth of *Streptomyces* sp. A8 was extracted with ethyl acetate at pH 3, purified by thin layer chromatography and then identified by HPLC chromatography. The result suggested that it appears to be an antibiotic belonging to the neomycin group (data not shown).

3.2. Ability to degrade organic compounds of Streptomyces sp. A8 strain

Production of extracellular enzymes to degrade organic compounds by *Streptomyces* sp. A8 was investigated. The result indicated that this strain was able to degrade starch,

protein and cellulose with the diameters of degradation zones were 12, 5 and 4 mm, respectively. This help to degrade the unconsumed feed and feces in the culture pond.

3.3. Antibiotic susceptibility of Streptomyces sp. A8

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 1, Fig. 6).

Antimicrobial agent	Zone diameter	Streptomyces sp. A8			
(Disk Content)	Resistant (R)	Intermediate (I)	Susceptible (S)		
Ampicillin (10 µg)	≤13	14-16	≥17	0	R
Erythromycin (15µg)	≤13	14-22	≥23	20.5	Ι
Chloramphenicol (30 µg)	≤ 12	13-17	≥ 18	24.0	S
Ciprofloxacin (5µg)	≤ 15	16-20	≥ 21	29.0	S
Tetracycline (30 µg)	≤ 14	15-18	≥ 19	13.0	R
Gentamicin (10 µg)	≤ 12	13-14	≥ 15	34.5	S
Penicillin- G (10 µg)	≤ 16	not suitable	≥ 17	0	R

Table 1. Antibiotic susceptibility of Streptomyces sp. A8



Fig. 6. Antibiotic sensitivity of Streptomyces sp. A8 strain.

3.4. Selection of some cultivative conditions and medium components for biomass production and antagonistic activity of Streptomyces sp. A8

The cultivative conditions for biomass production and antagonistic activity to V. parahaemolyticus V6 of Streptomyces sp. A8 were determined. In general, the selected 96 temperature was hours. Although Streptomyces sp. A8 was able to grow from pH 6 to 9 in SCB medium, the selected pH for and production of antagonistic growth component was 8. The highest biomass production and antagonistic activity were recorded in the SCB medium at 30°C.

The cultivative medium components for biomass production and antagonistic activity to *Vibrio parahaemolyticus* V6 of *Streptomyces* sp. A8 were investigated. The selected C-, N-, amino acid- and vitamin- sources were starch, casein, DL- α -alanine and vitamin B6, respectively. The selected concentrations of starch, casein, NaCl, DL- α -alanine and vitamin B6 were 13%, 0.6%, 16%, 0.6% and 0.02%, respectively.

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3.5. Fermentation for biomass production of Streptomyces sp. A8

Streptomyces sp. A8 was cultivated in the selected culture conditions and medium for biomass production and antagonistic activity of actinomy in flasks, 10-L fermenter and 120-L fermenter (Fig. 7). A large amount of actinomycete biomass (about 15.0 g/L) was harvested.



Fig. 7. Proliferation of Streptomyces sp. A8 biomass in flasks and fermenter.

4. Conclusions

Streptomyces sp. A8 isolated from shrimp pond sediment in Thua Thien showed the high activity against V. parahaemolyticus V6, which known as a mainly pathogenic agent of EMS, through production of inhibitory compounds and the ability to degrade organic compounds by production of extracellular enzymes. This strain was only resistant to several common antibiotics. When being fermented in selected culture conditions, a large amount of Streptomyces sp. A8 biomass was harvested. Therefore, Streptomyces sp. A8 can be considered as a promising candidate for control the shrimp diseases in industrial aquaculture.

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Nghiên cứu đặc tính của xạ khuẩn đối kháng với Vibrio parahaemolyticus được phân lập từ bùn ao nuôi tôm

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Tóm tắt: Hội chứng tôm chết sớm (EMS) hay Hội chứng hoại tử gan tụy cấp (AHPNS) gần đây đã được thêm vào danh sách các bệnh gây thiệt hại nghiêm trọng cho nuôi tôm tại Việt Nam. Việc phát triển một biện pháp phòng trị EMS hiệu quả là hết sức cấp thiết. Ở đây, chủng xạ khuẩn *Streptomyces* sp. A8 được phân lập từ bùn ao nuôi tôm đã thể hiện hoạt tính đối kháng cao với chủng vi khuẩn *Vibrio parahaemolyticus* V6 mà được coi là tác nhân chính gây EMS và khả năng phân hủy các hợp chất hữu cơ thường gây ô nhiễm cho môi trường ao nuôi tôm. *Streptomyces* sp. A8 chỉ kháng một số loại kháng sinh thông thường. Điều kiện nuôi cấy thích hợp cho sự hình thành sinh khối và hoạt tính đối kháng với *V. parahaemolyticus* V6 của *Streptomyces* sp. A8 là 96 giờ, pH 8 và 35°C trong môi trường SCB với nồng độ tinh bột, casein, NaCl, DL-α-alanine và vitamin B6 lần lượt là 13%; 0,6%; 16%; 0,6% và 0,02%. Khi lên men trong điều kiện tối ưu, một lượng lớn sinh khối *Streptomyces* sp. A8 đã được thu nhận.

Từ khóa: Xạ khuẩn, Streptomyces sp. A8, nuôi tôm, Vibrio parahaemolyticus, hội chứng tôm chết sớm.