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https://doi.org/10.5109/19533

出版情報：九州大学大学院農学研究院紀要. 56 (1), pp.23-31, 2011-02. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **56** (1), 23–31 (2011)

Identification and Characterization of *Pseudomonas* sp. P9 Antagonistic to Pathogenic *Vibrio* spp. Isolated from Shrimp Culture Pond in Thua Thien Hue–Viet Nam

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(*Received October 29, 2010 and accepted November 8, 2010*)

From shrimp culture ponds in Thua Thien Hue, isolates were obtained on PSA plates and screened for their activity against two pathogenic strains *Vibrio* sp. V7 and V10 isolated from diseased shrimps in culture ponds in Thua Thien Hue using the agar well–diffusion method. Among of the isolates, only strain P9 showed antagonistic activity against *Vibrio* sp. V7 and V10. Based on the cell morphology observed with a JEOL 5410 LV scanning electron microscope, biochemical reactions tested with GN card, and 16S rRNA nucleotide sequence used to search the GenBank database with the BLAST system, strain P9 was identified as *Pseudomonas* sp. P9. The 16S rRNA sequence of *Pseudomonas* sp. P9 has been deposited in the DDBJ/EMBL/GenBank database with the accession number HM854226. In addition, *Pseudomonas* sp. P9 also showed the ability to produce siderophore to acquire iron and extracellular enzymes to decompose organic compounds such as starch, protein and cellulose. Otherwise *Pseudomonas* sp. P9 was non–pathogenic to shrimp. Growth as well as production of the antagonistic component in TSBS of *Pseudomonas* sp. P9 showed over a wide range of pH, salinities and temperatures. Therefore, *Pseudomonas* sp. P9 can be considered as not only a candidate for control the shrimp disease but also a potential probiont for shrimp culture.

Keywords: antagonism, biocontrol, probiotic, *Pseudomonas*, shrimp ponds, *Vibrio*, Vibriosis

INTRODUCTION

Shrimp production has been affected severely by infectious diseases caused by *Vibrio* spp. (Gomez *et al*., 2000; Vijayan *et al*., 2006). Antibiotics are used widely to control vibriosis, but have a serious negative impact to the environment. Residual antibiotics in the environment increase the resistance in aquatic bacteria and change the bacterial flora in sediment (Lalumera *et al*., 2004). As the resistance determinant can be easily transferred to terrestrial animal and human bacterial pathogens, aquaculture is one of the sources of antibiotic resistance in terrestrial animal and human bacterial pathogens (Cabello, 2006). In fact, many pathogenic *Vibrio* have been determined to be resistant to many available antibiotics (Tom *et al*., 2007). In addition, the use of antibiotic as prophylactic agents in feed results in residues in shrimp product (Esposito *et al*., 2007). The residual antibiotics may cause toxicity, allergic reactions and alteration of normal microflora of consumer (Sinhaseni *et al*., 2000). Therefore, the development of an alternative measure to control the proliferation of pathogenic vibrios is urgent. The most successful and acceptable measure has been reported to be the use of probiotics as biological control agents in shrimp aquaculture.

Members of the genus *Pseudomonas* are attractive bacteria for testing as sources of new bioactive compounds including antibiotics, bacteriocins, biosurfactants and bacteriolytic enzymes (Vijayan *et al*., 2006; Kumar *et al*., 2008; Sivaprakasam *et al*., 2008). They are widely distributed in soil and aquatic habitats including shrimp ponds (Sakami *et al*., 2008). There have been reports that *Pseudomonas* spp. produce bioactive compounds with ability to control vibrios such as *V. harveyi* and *V. parahaemolyticus*, and that have no effect on the shrimp (Gram, 1993; Chythanya *et al*., 2002; Vijayan *et al*., 2006; Rattanachuay *et al*., 2007).

Hence, the aims of this study were to screen *Pseudomonas* strain antagonistic to pathogenic *Vibrio* strains from shrimp culture ponds in Thua Thien Hue and investigate its probiotic characteristics. These results may facilitate studies which offer the potential application of probiont for improving the shrimp aquaculture in Thua Thien Hue (Viet Nam).

MATERIALS AND METHODS

Sample Collection and Processing

Water, sediment and shrimp samples were collected from shrimp culture ponds located at Phu Loc (16°13'37"N, 108°02'40"E), Phu Vang (16°31'12" N, 107°38'38"E), and Quang Dien (16°34'08"N, 107°33'44"E), Thua Thien Hue, Viet Nam. Six random samples of water and sediment from each culture ponds were collected aseptically in sterile plastic bottles and bags, respectively. All samples were 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. Water and sediment samples were stored at 4 °C. The shrimp samples were rinsed with sterile physiological saline, followed by a dis-

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tilled water wash. Hepatopancreas of diseased shrimps and intestinal tracts of healthy shrimps (*Penaeus monodon*) were drawn aseptically and homogenised in physiological saline. These samples were immediately used for isolation of strains *Vibrio* and *Pseudomonas*, respectively.

Isolation, identification and characterization of pathogenic Vibrio spp.

Vibrio spp. were isolated from 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 ºC for 24–48 hours and maintained 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. Shrimps 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 isolates 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 ºC, followed by 35 cycles of 1 min at 95 ºC, 55 sec at 58 ºC and 1 min at 72 ºC, and a final extension of 7 min at 72 ºC. The 16S rRNA nucleotide sequences were compared with available 16S rRNA 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 National Committee for Clinical Laboratory Standards (NCCLS).

Isolation of Pseudomonas strains

Bacteria were isolated on Pseudomonas Selective Agar (PSA, Merck, Germany) medium from water, sediment, and healthy shrimp samples using the spread plate method. Culture plates were incubated at 30 °C for 24–48 hours. Typical colonies of genus *Pseudomonas* from each sample were selected, streaked on PSA containing 1.5% NaCl and incubated at 30 ºC. The obtained pure strains were maintained at 4 ˚C on PSA slants with 1.5% NaCl.

Antagonism assay

Activities against pathogenic *Vibrio* strains of isolates were determinated by the agar well–diffusion method. The cultures of the *Vibrio* strains were prepared by pouring 0.1 mL of the inoculum onto TCBS (HiMedia, India) plates, streaking with a swab over the entire surface of the agar and air–drying for 15 min in an incubator set at 30 °C. Six–mm diameter wells were punched into the agar using pipette tips, which were cut to obtain a 6 mm diameter bore and then sterilized. The isolates were inoculated in tryptone soya broth (TSB, Becton Dickinson, USA) containing 1.5% NaCl and incubated at 30 °C for 48 hours on a shaker at 150 rpm. The culture supernatants were obtained by centrifuging at 3,000 rpm, 4 °C for 15 min. One hundred microlitres of culture supernatant was carefully pipetted into each well. The resulting plates were placed at 4 ˚C for 12 hours and incubated for 18–24 hours at 35 ˚C. The diameters of the inhibition zones around the wells were then recorded in millimeters.

Identification of Pseudomonas isolate

Morphological characteristics

Colony of *Pseudomonas* isolate which appeared on the PSA (Merck, Germany) at 30 ˚C, 24 hours of incubation was characterized based on its size, shape and color. Besides, the culture grown tryptone soya broth (TSB, Becton Dickinson, USA) was centrifuged, and the pellet was 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 sample was rinsed gently with deionized water and dehydrated then 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, observed with a JEOL 5410 LV scanning electron microscope.

Biochemical characteristics

Biochemical characteristics of *Pseudomonas* isolate were determined using the VITEK 2 GN card (bioMérieux, France) based on established biochemical methods and newly developed substrates measuring carbon source utilization, enzymatic activities, and resistance. There are 47 biochemical tests and one negative control well. Final identification results are available in approximately 10 hours or less.

Phylogenetic characteristics

The phylogenetic characteristics were determined based on the base sequences of 16S rRNA. DNA extraction of isolates was conducted following protocol of Sambrook and Russell (2001). PCR reactions were carried out in a final volume of 25μl 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). DNA primers were used in the forward, 341F 5'–CCTACGGGAGGCAGCAG–3') with a GC clamp (5'–CGCCCGCCGCGCCCCGCGCCC–GTCCCGCCGCCCCCGCCCG–3') and reverse, 907R (5'–CCGTCAATTCCTTTRAGTTT–3'). The amplifications were programmed for an initial denaturation of 5 min at 95 ºC, followed by 35 cycles of 1 min at 95 ºC, 55 sec at 58 ºC and 1 min at 72 ºC, and a final extension of 7 min at 72 ºC. The amplified product was purifed with Qiaquick® Mini Columns (Qiagen–German). ABI PRISM 3100–Avant

Genetic Analyzer (Applied Biosystems, USA) and ABI PRISM 3100–Avant Genetic Analyzer Data Collection v1.0 software were used for sequence analysis. The 16S rRNA nucleotide sequence was compared with available 16S rRNA sequences in GenBank using the BLAST system (Altschul *et al.*, 1990) and submitted to DDBJ/EMBL/GenBank to get respective accession number.

Extracellular enzymes production

Extracellular enzymes production (amylase, protease and cellulase) of *Pseudomonas* isolate were tested on Nutrient Agar (NA, Difco, USA) plates containing separately 1% starch, 1% carboxymethyl cellulose (CMC), and 1% casein. *Pseudomonas* isolate was grown on tryptone soya broth (TSB, Becton Dickinson, USA) containing 1.5% NaCl (TSBS) for 24 hours at 30 ˚C, 150 rpm. The culture supernatant of *Pseudomonas* strain was obtained by centrifuging at 3,000 rpm, 4 °C for 15 min. Wells were punched into the center of agar plates using sterilized bore. Seventy microliters of the culture supernatant was carefully pipetted into each well. The plates were kept at 4 ˚C for 4–6 hours and incubated at 30 ˚C for 24 hours. After that, the starch or CMC– contained plates were flooded with Lugol's iodine solution. A zone of clearance around the well indicated production of amylase and cellulase, respectively. Whereas, 10% mercuric chloride reagent were flooded over the casein–contained plates. A zone of clearance around the well indicated production of protease. The diameters of the clearance zones were recorded in millimeters.

Siderophore production

Production of siderophores by the *Pseudomonas* isolate was determined using blue agar (chrome azurol S or CAS agar) and CAS assay solutions in cell–free culture supernatants (Payne, 1994)

CAS agar plates. To prepare 1 liter of blue agar, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (Ⅲ) solution (1 mM $FeCl_3$. $6H_2O$, 10 mM HCl). Under stirring this solution was slowly added to 72.9 mg HDTMA dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. Also autoclaved was a mixture of 750 ml H_2O, 100 ml 10X MM9 salts, 15 g agar, 30.24 g Pipes, and 12.00 g of a 50% (w/w) NaOH solution to raise the pH to 6.8. After cooling to 50 °C, 30 ml casamino acids (10%), 0.2% glucose, 0.095% $MgCl_2$, 0.011% $CaCl_2$ were added as sterile solutions. The dye solution was finally added along the glasswall, with enough agitation to achieve mixing without generation of foam. Each plate received 30 ml of blue agar.

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 24 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 test

Pathogenicity of Pseudomonas isolate to Penaeus monodon larvae

Pathogenicity of *Pseudomonas* isolate was tested on the larvae of *P. monodon* (stage PL 16). Fifty PL were distributed in 20 L plastic basins containing 10 L sterile seawater, and fed with sterile commercial powder feed. The isolate culture grown at 35 °C on TSA medium containing 1.5% NaCl for 24 h was scraped from the surface of the plates using a sterile loop and suspended in sterile saline to McFarland 0.5, corresponding to 10^8 cfu/ml. The shrimp larvae were challenged at 10^6 cfu/ml. Shrimp larvae were grown in sterile saline alone served as control. The larval mortality was monitored for up to 5 days.

Pathogenicity of Pseudomonas isolate to juvenile tiger shrimps

Shrimps (4–5 g) were challenged by injecting intramuscularly with 10^7 cfu/g of *Pseudomonas* isolate in the site between the third and fourth abdominal segment (Vera *et al.*, 1992). Control shrimp were injected with sterile saline. A total of 10 shrimps was grown in plastic basins (20–L capacity) containing 10–L sterile seawater and fed with the commercial pellet feed. The mortalities of juvenile tiger shrimps were observed for up to 5 days.

Antibiotic susceptibility of Pseudomonas sp. P9

The antibiotic susceptibility was performed by disk diffusion method established by Bauer *et al.* (1966) and standardized by 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 (10 μg), penicillin–G (10 μg) were employed. The bacterial 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 millimeters and compared with the standard interpretative chart to determine their antibiotic sensitivity. For quality control, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were tested under the same conditions and antibiotic agents. The zone sizes shown by the control organisms should fall within the range of diameters given by NCCLS.

Optimal temperature, pH, salinity and culture time for the growth and antagonistic action of *Pseudomonas* isolate

The optimal conditions for growth and production of antagonistic action of *Pseudomonas* isolate was assessed by growing on TSBS at various pH (4.0, 5.0, 6.0, 7.0, 8.0

and 9.0), supplemented sodium chloride (0, 1, 2, 3, 4 and 5%), temperatures (20, 25, 30, 35 and 40 °C), and culture time (1, 2, 3 and 4 days). The growth of *Pseudomonas* isolate was estimated via absorbance of culture at 600 nm using a spectrophotometer. The activities against pathogenic *Vibrio* strains of *Pseudomonas* isolate were determined by the agar well–diffusion method with 70 μl of culture supernatant was pipetted into each well.

Statistical analysis

Three replicates were used in each experiment, unless otherwise stated. All results were presented as means ± their standard deviations. A one–way ANOVA (SPSS for windows version 16.0) was used to analyze statistical differences at a P–value < 0.05 and mean comparisons were performed by the Duncan's multiple range test.

RESULTS

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 reached a 98.5% identity 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 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 and HM854228, respectively.

Antibiotics sensitivities tests revealed *Vibrio* sp. V7 and *Vibrio* sp. V10 to be sensitive 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), whereas *Vibrio* sp. V7 was intermediate to ampicillin (Amp) and gen-

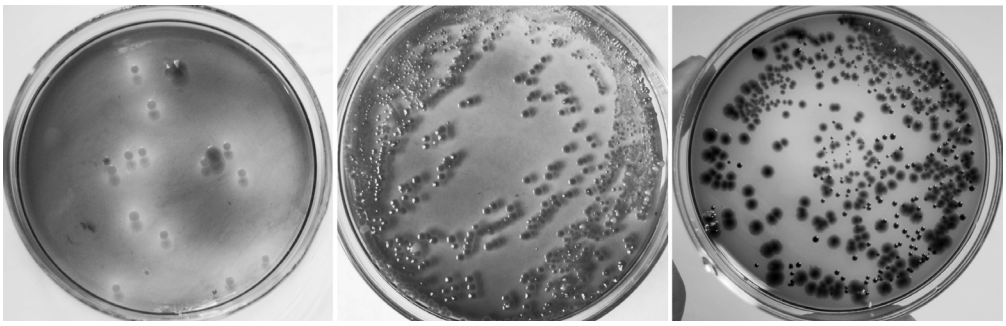

Fig. 1. Colonial morphology of *Vibrio* spp. isolated from Lang Co–Phu Loc (left), Quang An–Quang Dien (middle) and Loc Dien–Phu Loc (right) on TCBS at 35 °C, 24–48 h.

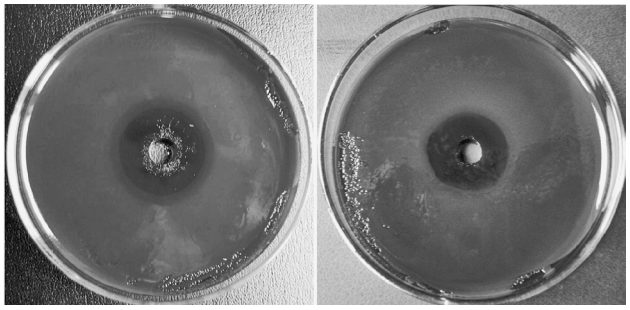

Fig. 2. Activity against strain V7 (left) and V10 (right) of strain P9.

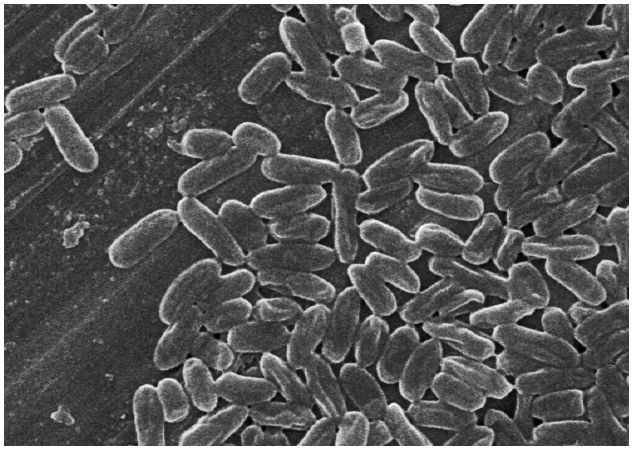

Fig. 3. Microscopic characteristic of strain P9.

tamicin (Gen).

Isolation of *Pseudomonas* strains

A total of 41 strains of *Pseudomonas* spp. was isolated on Pseudomonas Selective Agar from the water, sediment and shrimp samples collected at shrimp culture ponds in Thua Thien Hue. These strains were coded from P1 to P41.

Activities against pathogenic *Vibrio* spp. of *Pseudomonas* isolates

All *Pseudomonas* isolates were screened for their activities against two strains *Vibrio* sp. V7 and V10 using the agar well diffusion method. Of these, strain P9 showed the highest activity against both strains V7 and V10 (Fig. 2). The diameters of zone antagonistic to strains V7 and V10 were 26 and 25 mm, respectively. Therefore, strain P9 was selected for further studies.

Table 1. Test reactions on GN Card of strain P9

Well	Test	Mnemonic	Amount/well (mg)	Reaction
2	Ala–Phe–Pro–ARYLAMIDASE	APPA	0.0384	–
3	ADONITOL	ADO	0.1875	–
4	L–Pyrrolydonyl–ARYLAMIDASE	PyrA	0.018	–
5	L–ARABITOL	IARL	0.0234	–
7	D–CELLOBIOSE	Dcel	0.3	–
9	BETA–GALACTOSIDASE	BGAL	0.036	–
10	H2S PRODUCTION	H2S	0.0024	–
11	BETA–N–ACETYL–GLUCOSAMINIDASE	BNAG	0.0408	–
12	Glutamyl Arylamidase pNA	AGLTp	0.0324	–
13	D–GLUCOSE	DGLU	0.3	+
14	GAMA–GLUTAMYL–TRANSFERASE	GGT	0.0228	+
15	FERMENTATION/ GLUCOSE	OF	0.45	–
17	BETA–GLUCOSIDASE	BGLU	0.036	–
18	D–MALTOSE	dMAL	0.3	–
19	D–MANNITOL	dMAN	0.1875	–
20	D–MANNOSE	dMNE	0.3	+
21	BETA–XYLOSIDASE	BXYL	0.0324	–
22	BETA–Alanine acrylamidase	BAlap	0.0174	+
23	L–Proline ARYLAMIDASE	ProA	0.0234	+
26	LIPASE	LIP	0.0192	+
27	PALATINOSE	PLE	0.3	–
29	Tyrosine ARYLAMIDASE	TyrA	0.0276	–
31	UREASE	URE	0.15	+
32	D–SORBYTOL	dSOR	0.1875	–
33	SACCHAROSE/SUCROSE	SAC	0.3	–
34	D–TAGATOSE	dTAG	0.3	–
35	D–TREHALOSE	dTRE	0.3	–
36	CITRATE (SODIUM)	CIT	0.054	+
37	MALONATE	MNT	0.15	+
39	5–KETO–D–GLUCONATE	5KG	0.3	–
40	L–LACTATE alkalinisation	ILTAk	0.15	+
41	ALPHA–GLUCOSIDASE	AGLU	0.036	–
42	SUCCINATE alkalinisation	SUCT	0.15	+
43	Beta–N–ACETYL GALACTOSAMINIDASE	NAGA	0.0306	–
44	ALPHA–GALACTOSIDASE	AGAL	0.036	–
45	PHOSPHATASE	PHOS	0.0504	–
46	Glycine ARYLAMIDASE	GlyA	0.012	–
47	ORNITHINE DECARBOXYLASE	ODC	0.3	–
48	LYSINE DECARBOXYLASE	LDC	0.15	–
52	DECARBOXYLASE BASE	ODEC	N/A	
53	L–HISTIDINE assimilation	IHISa	0.087	–
56	COUMARATE	CMT	0.126	+
57	BETA–GLUCORONIDASE	BGRU	0.0378	–
58	O/129 RESISTANCE (comp.vibrio)	O129R	0.0105	+
59	Glu–Gly–Arg–ARYLAMIDASE	GGAA	0.0576	–
61	L–MALATE assimilation	IMLTa	0.042	+
62	ELLMAN	ELLM	0.03	–
64	L–LACTATE assimilation	ILATa	0.186	+

Identification of strain P9

Morphological characteristics

Pseudomonas Selective Agar (PSA, Merck, Germany) is a selective and differential medium for the isolation of *Pseudomonas* species. Colonies of strain P9 are small, rough and surrounded by a blue–green pigment on PSA medium. Strain P9 is a Gram–negative rod, 0.5–0.8 × 1.5–3.0 μm and motile (Fig. 3).

Biochemical characteristics

Biochemical characteristics of strain P9 determined using the VITEK 2 GN card (bioMérieux, France) was shown in Table 1.

Phylogenetic characteristics

The 16S rRNA nucleotide sequence of strain P9 was determined and shown in Fig. 4. The result of the homology search with GeneBank database using the BLAST system indicated that the 16S rRNA nucleotide sequence of strain P9 had a 100% identity with that of *Pseudomonas aeruginosa* TERIPS9006; 97.9% with that of *Pseudomonas* sp. P60; and 97% with that of *P. aeruginosa* SML. Thereby, strain P9 was considered to belong genus *Pseudomonas* and identified as *Pseudomonas* sp. P9. The 16S rRNA nucleotide sequence of *Pseudomonas* sp. P9 have been deposited in the DDBJ/EMBL/GenBank database with the accession number HM854226.

Extracellular enzymes production

Production of extracellular enzymes to degrade organic compounds, such as starch, protein and cellulose by *Pseudomonas* sp. P9 was investigated and presented in Fig. 5. 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.

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  1 GCGAAGCCTG ATCCAGCCAT GCCGCGTGTG TGAAGAAGGT CTTCGGATTG TAAAGCACTT
 61 TAAGTTGGGA GGAAGGGCAG TAAGTTAATA CCTTGCTGTT TTGACGTTAC CAACAGAATA
121 AGCACCGGCT AACTTCGTGC CAGCAGCCGC GGTAATACGA AGGGTGCAAG CGTTAATCGG
181 AATTACTGGG CGTAAAGCGC GCGTAGGTGG TTCAGCAAGT TGGATGTGAA ATCCCCGGGC
241 TCAACCTGGG AACTGCATCC AAAACTACTG AGCTAGAGTA CGGTAGAGGG TGGTGGAATT
301 TCCTGTGTAG CGGTGAAATG CGTAGATATA GGAAGGAACA CCAGTGGCGA AGGCGACCAC
361 CTGGACTGAT ACTGACACTG AGGTGCGAAA GCGTGGGGAG CAAACAGGAT TAGATACCCT
421 GGTAGTCCAC GCCGTAAACG ATGTCGACTA GCCGTTGGGA TCCTTGAGAT CTTAGTGGCG
481 CAGCTAACGC GATAAGTCGA CCGCCTGGGG AGTACGGCCG CAAGGTTAAA ACTCCGGGGG
541 AATTGGACGG AA
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Fig. 4. The 16S rRNA nucleotide sequence of strain P9.

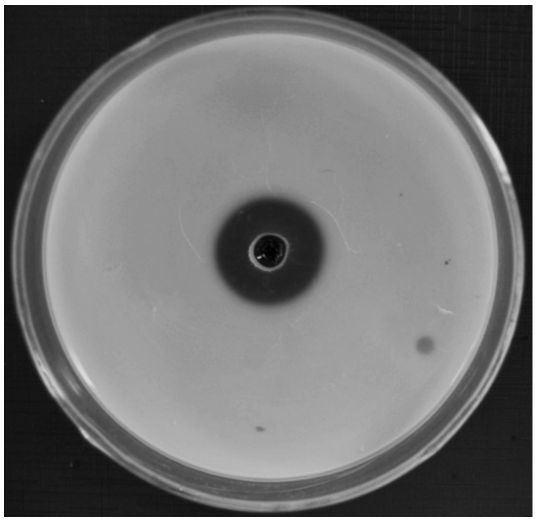

Fig. 5. Degradation of casein of *Pseudomonas* sp. P9.

Siderophore production

Pseudomonas sp. P9 produced siderophores on CAS agar as indicated by a yellowish halo around the colonies, indicating their ability to survive and grow in the iron deficient conditions. The organism was found to produce 50% siderophore units as detected by the CAS assay technique (Fig. 6).

Pathogenicity to larvae and juvenile tiger shrimps (*P. monodon*)

The pathogenicity of *Pseudomonas* sp. P9 test on larvae and juvenile tiger shrimps (*P. monodon*) indicated that this strain did not cause any significant mortality after 5 days of challenge at 10^6 cfu/ml and 10^7 cfu/g, respectively.

Antibiotic susceptibility

Antibiotics sensitivities tests revealed *Pseudomonas* sp. P9 to be sensitive to ciprofloxacin (Cip) and gentamicin (Gen), intermediate to chloramphenicol (Chl), and resistant to ampicillin (Amp), erythromycin (Ery), tetracycline (Tet) and penicillin–G (Pen) (Table 2, Fig. 7).

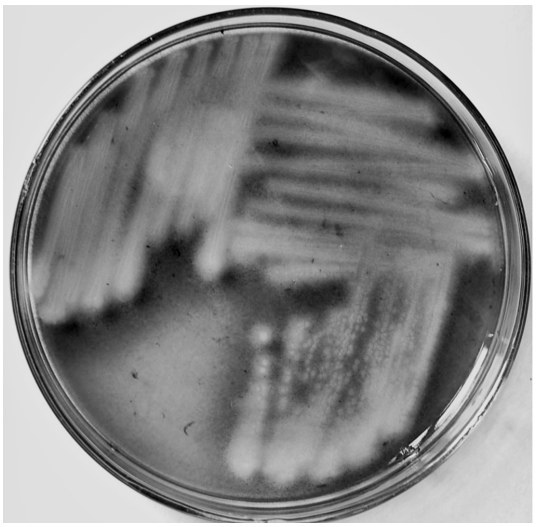

Fig. 6. Siderophore production of *Pseudomonas* sp. P9 on CAS agar.

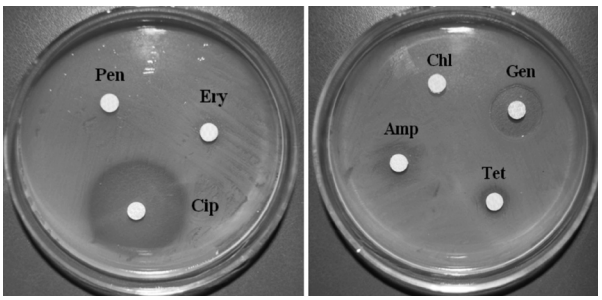

Fig. 7. Antibiotics sensitivities of *Pseudomonas* sp. P9.

Table 2. Zone diameter interpretive standards and antibiotic susceptibility of *Pseudomonas* sp. P9

Antimicrobial agent (Disk Content)	Diameter of inhibition zone (mm)			*E. coli* ATCC 25922	*S. aureus* ATCC 25923	*Pseudomonas* sp. P9	
	Resistant (R)	Intermediate (I)	Susceptible (S)				
Ampicillin[a] (10 μg)	not effective (naturally resistant)			23	35	8	R
Erythromycin[c] (15 μg)	$\leq$ 13	14 – 22	$\geq$ 23	10	30	7	R
Chloramphenicol[a] (30 μg)	$\leq$ 12	13 – 17	$\geq$ 18	26	26	13	I
Ciprofloxacin[b] (5 μg)	$\leq$ 13	14 – 22	$\geq$ 23	40	28	38	S
Tetracycline[a] (25 μg)	$\leq$ 14	15 – 18	$\geq$ 19	25	28	11	R
Gentamicin[a] (15 μg)	$\leq$ 12	13 – 14	$\geq$ 15	20	22	20	S
Penicillin–G[d] (10 μg)	$\leq$ 16	not suitable	$\geq$ 17	7	37	7	R

Source: National Committee for Clinical and Laboratory Standard. 2001. "*Performance standards for antimicrobial susceptibility testing. Eleventh Informational Supplement*". M100-S11.

[a] Criteria for interpretation of susceptibility of *Pseudomonas auroginosa*.

[b] Criteria for interpretation of susceptibility of *Enterobacteriaceae*.

[c,d] Criteria for interpretation of susceptibility of *Enterococcus* spp.

Optimal temperature, pH, salinity and culture time for growth and antagonistic activity

Optimal culture conditions for growth and antagonistic activity to *Vibrio* sp. V7 of *Pseudomonas* sp. P9 were presented in Fig. 8. Although *Pseudomonas* sp. P9 was able to grow from pH 6 to 10 in TSBS medium, the optimum pH for growth recorded for the production of the antagonistic component was 6.0 after 48 hours. The highest growth and antagonistic activity were attained after 48 hours in the broth supplemented 1% NaCl. *Pseudomonas* sp. P9 showed optimum growth and antagonistic action at 35 °C. Production of the antagonistic component was found after about 24 hours of growth when the culture entered the late log phase, and the activity peaked in the stationary phase and remained stable thereafter, with only minor fluctuations in the activity. The maximum inhibitory zone of 14 mm was observed at optimum culture conditions.

DISCUSSION

In the present study, a putative probiont, *Pseudomonas* sp. P9, was isolated from shrimp sample collected from a brackish water pond located at Lang Co, Phu Loc, Thua Thien Hue (Viet Nam). *Pseudomonas* sp. P9 has showed antagonistic characteristic towards both pathogenic strains, *Vibrio* sp. V7 and V10, isolated from diseases shrimps collected from culture ponds in Thua Thien Hue. *Vibrio* sp. V7 and V10 were resistant to penicillin and ampicillin. In fact, it is reported that many

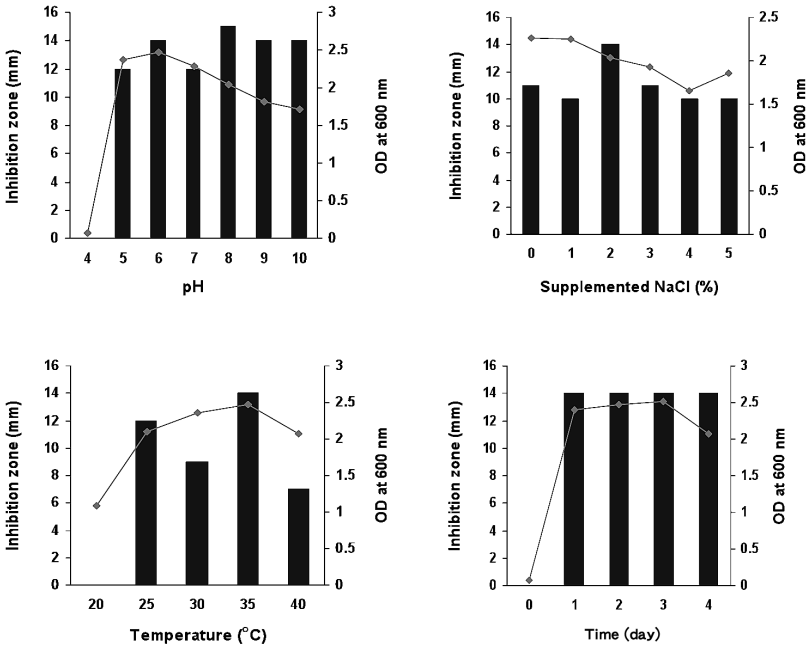

Fig. 8. Effect of some culture conditions on growth (line) and antagonistic activity against *Vibrio* sp. V7 (column) of *Pseudomonas* sp. P9.

pathogenic *Vibrio* have been determined to be resistant to many available antibiotics (Tom *et al*., 2007). The emergence of antibiotic–resistant pathogenic *Vibrio* make the development of alternative counter measures against vibriosis is of global concern. One of the promising counter measures is the use of biocontrol agent in marine aquaculture (Alim *et al*., 2009). *Pseudomonas* spp. and vibrios are the most common genera associated with crustaceans (Moriarty, 1997) and are common inhabitants of the aquatic environment including shrimp culture ponds (Otta and Karunasagar, 1999). Certain characteristics, such as the ability to produce adhesins, inhibitory substances like bacteriocines, antibacterial substances and siderophores, competition with pathogens for chemicals and energy, and the ability to boost the immune response, in addition to being non–pathogenic to the target animal have been suggested as traits required in an organism to be a candidate probiotic (Verschuere *et al*., 2000). Here, *Pseudomonas* sp. P9 were screened for desirable traits such as the ability to produce inhibitory substances, secrete extracellular macromolecule digesting enzymes, and produce iron sequestering compounds like siderophores. As far as you concern, *Pseudomonas* are attractive bacteria for testing as sources of new bioactive compounds including antibiotics, bacteriocins, biosurfactants and bacteriolytic enzymes (Raaijmakers *et al*., 1997; Gram *et al*., 1999; Vijayan *et al*., 2006; Rattanachuay *et al*., 2007; Kumar *et al*., 2008 and Sivaprakasam *et al*., 2008). Iron is a limiting bioactive metal in seawater and essential for the growth of marine bacteria. Competition for iron is also a possible mechanism for aquaculture probiotics to control the pathogens. Numerous studies have implicated siderophores as bacteriostatic substances produced by *Pseudomonas* species (Guerinot, 1994 and Vijayan *et al*., 2006) and the inhibitory activity of many *Pseudomonas* strains seemed indeed mediated by siderophores (Gram, 1993 and Gram *et al*., 1999). Thus, the siderophore–producting *Pseudomonas* strains could influence the growth of pathogenic *Vibrio* spp. by competition for iron in marine water. Besides, in order to be considered as a probiotic for application in shrimp culture systems, the strain has to be evaluated for safety to the host (Verschuere *et al*., 2000 and Singh *et al*., 2003). In this case, the morphological, biochemical and phylogenetic characteristics of *Pseudomonas* sp. P9 were quite similar to *Pseudomonas auroginosa* species, however did not cause any harmful effects to shrimp larvae or sub–adults upon challenge even at a density of 10^7cfu/g introduced by injection. Also, specific inhibition of *Vibrio harveyi* by *Pseudomonas aeruginosa* has been reported earlier by Torrento and Torres (1996), Chythanya *et al*. (2002) and Vijayan *et al*. (2006). On the other hand, the production pattern of the antagonistic component in TSBS medium of *Pseudomonas* sp. P9 showed an increase in the late stationary phase and this was sustained over 4 days in the supernatant. Growth as well as production of the antagonistic component in TSBS of *Pseudomonas* sp. P9 over a wide range of pH, salinities and temperatures suggests that the isolate could be a suitable candidate probiotic for both penaeid and non–penaeid systems.

In conclusion, based on the unique characteristics, such as, a significant antibacterial property towards bacterial pathogens, and tolerance to a wide range of physico–chemical conditions of shrimp rearing systems, production of extracellular enzymes, as well as safety to larvae and juvenile shrimps, *Pseudomonas* sp. P9 can be considered as not only a candidate for control the shrimp diseases but also a potential probiont for shrimp culture.

ACKNOWLEDGEMENTS

This study was funded by the Vietnam's National Foundation for Science and Technology Development (NAFOSTED).

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