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Vibrio-inhibiting Marine Bacteria Isolated from Black Tiger Shrimp Hatchery

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Forty five strains of marine bacteria which showed inhibitory activities against bacterial swarming were isolated from larval black tiger shrimp (*Penaeus monodon*), brine shrimp (*Artemia salina*) nauplii and rearing seawater in Thailand. These marine bacteria were examined by a double-layer plate method for their growth-inhibiting abilities against 50 strains of vibrios isolated from similar sources. As a result, 27 strains among 45 marine bacteria examined inhibited 32 to 94% of the vibrios tested. These *Vibrio*-inhibiting strains also exhibited inhibitory activities against 52 to 96% of the reference strains of 27 species of *Vibrio* including fish and shellfish pathogens. The inhibition was exhibited bactericidally, and antibiotics were produced extracellularly and inactivated by heat treatment at 70°C for 30 min. All the 27 strains of antibiotic producers were identified as *Alteromonas*-like organisms based on their taxonomical characteristics including G + C values of DNA (42.4–45.3 mol %).

Key words: *Alteromonas*, anti-*Vibrio* substance, black tiger shrimp, *Penaeus monodon*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*

The success in mass seed production of black tiger shrimp (*Penaeus monodon*) and the advances in culture technology have accelerated the development of shrimp farming industry in Thailand over the past 15 years (Tansutapanich *et al.*, 1990; Sritongsuk and Tookvinas, 1991). Millions of shrimp postlarvae produced annually by government and private hatcheries have been supplied as seedlings for commercial culture in growout ponds. The success in shrimp propagation has prompted the Thai Department of Fisheries to plan a massive program to stock coastal waters to replenish the declining natural standing crops. The seed production techniques of marine shrimp have been apparently well established. However, this establishment has not been sustained without problems; diseases caused by various pathogens have inflicted high mortalities of shrimp larvae on many occasions (Ruangpan, 1981, 1985; Flegel *et al.*, 1992)

Vibrio infections, especially luminous bacterial disease caused by *V. harveyi*, were the most notorious in shrimp larvae (Flegel *et al.*, 1992). Some other species like *V. parahaemolyticus*, *V. fluvialis*, *V. alginolyticus* and *Vibrio* spp. were detected in greater number from moribund black tiger shrimp larvae than normal ones (Tanasomwang and Ruangpan, 1995). Antibiotics and/or chemicals have been widely used for prophylactic and therapeutic purposes as a consequence of the frequent outbreaks of these diseases. Nevertheless, the sensitivity test of 4 genera of bacteria against 9 antimicrobial agents revealed that efficacy of these drugs against bacteria especially vibrios was much reduced when they were used in seawater (Tanasomwang and Supaiwan, 1996). Chemicals such as formalin, benzalkonium chloride and povidone iodine could inhibit the growth of various species of *Vibrio* only at quite high concentrations (Tanasomwang and Supaiwan, 1997). The effectiveness of the drugs and/or chemicals in seawater seemed to be limited.

In the present study, some marine bacteria among the flora of black tiger shrimp larvae, brine shrimp (*Artemia salina*) and seawater were found to be antagonistic to

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Vibrio species which were isolated from the similar sources and various known species of *Vibrio* including fish and shellfish pathogens. This finding suggests the possible use of these antibiotic-producing bacteria for controlling the disease outbreaks in seed production process.

Materials and Methods

Isolation of marine bacteria inhibiting bacterial swarming

Forty five strains of marine bacteria able to inhibit swarming of bacteria were isolated; 22 from normal black tiger shrimp larvae, 16 from brine shrimp nauplii and 7 from seawater of shrimp tank and coast. These strains were isolated by the following procedure. Groups of 20 hatchery-produced black tiger shrimp larvae or 0.1 g wet weight of brine shrimp nauplii were homogenized in glass homogenizers each containing 2 m*l* or 0.9 m*l* of sterile seawater, respectively. Samples (0.1 m*l*) of serially 10-fold diluted suspensions were spread onto Marine Agar 2216 (MA, Difco Laboratories). Undiluted and 10-fold diluted 0.1 m*l* of seawater samples were spread onto the same medium. After incubating at 30°C for 48 h, swarming bacteria naturally appeared on the plates, and the colonies which formed clear zones of inhibition against such bacterial swarming were picked up and purified on MA. These bacteria were inoculated in semisolid MA and stored at 20°C for further studies.

Vibrio *strains*

Fifty strains of *Vibrio* that have been isolated and identified according to the scheme described by Muroga *et al.* (1987) and Bergey's Manual of Systematic Bacteriology Vol. 1 (Krieg and Holt, 1984) were used in this investigation (Table 1). Thirty one of these were isolated from both healthy and diseased shrimp larvae, rearing water and brine shrimp nauplii in the previous study (Tanasomwang and Ruangpan, 1995). The remaining were supplementally isolated for the present study; 9 from brine shrimp nauplii and 10 from luminous diseased shrimp larvae. In addition, reference strains of 27 species of the genus *Vibrio* including fish and shellfish pathogens were also used in this study; these were *V. aestuarianus* ATCC35048, *V. alginolyticus* HUFP9107 (= NCMB1903), *V. anguillarum* HUFP5001 (= ATCC19264), *V. campbellii* HUFP9109 (= ATCC25920), *V. carchariae* HUFP9110 (= ATCC35084), *V. cholerae* non-O1 PS-7702, *V. cincinnatiensis* ATCC35912, *V. damsela* (= *Photobacterium damsela*) ATCC33539, *V. diazotrophicus* HUFP9301 (=ATCC33466), *V. fisheri* ATCC7744, *V. fluvialis* HUFP9302 (= NCTC11327), *V. gazogenes* HUFP9303 (= ATCC29988), *V. harveyi* HUFP9111 (= ATCC14126), *V. ichthyoenteri* F-2, *V. mediterranei* ATCC43341, *V. natriegens* ATCC14048, *V. navarrensis* ATCC51183, *V. nereis* HUFP9112 (= ATCC25917), *V. nigripulchritudo* ATCC27043, *V. ordalii* ATCC33509, *V. parahaemolyticus* HUFP9114 (= ATCC17802), *V. pelagius* HUFP9115 (= ATCC25916), *V. penaeicida* KH-1, *V. proteolyticus* HUFP9307 (= NCMB1326), *V. splendidus* HUFP9117 (= ATCC33125), *V. tubiashii* HUFP9118 (= ATCC19109), *V. vulnificus* HUFP5002 (= ATCC27562).

Table 1. Fifty strains of *Vibrio* isolated from black tiger shrimp hatchery used in this study

Species	Number of strain	Source
Vibrio parahaemolyticus	2	Diseased shrimp larvae
	1	Rearing water
Vibrio fluvialis	4	Diseased shrimp larvae
	1	Brine shrimp
Vibrio alginolyticus	1	Diseased shrimp larvae
	4	Rearing water
	3	Brine shrimp
Vibrio harveyi	10	Luminous diseased shrimp larvae
Vibrio spp.	3	Normal shrimp larvae
	8	Diseased shrimp larvae
	8	Brine shrimp
	5	Rearing water

Inhibition test

Forty five strains of anti-swarming bacteria were tested for their potential in inhibition of *Vibrio* strains by using a double-layer plate method (Imada *et al.*, 1985; Dopazo *et al.*, 1988). Plates of MA were stab-inoculated with the anti-swarming bacteria. Each plate could be inoculated up to 7 strains. After incubating at 30°C for 4 days, the bacteria were killed with chloroform vapour for 10 min and overlaid with MA containing a small amount of each strain of *Vibrio*. The plates were further incubated at 30°C for 2 days. A clear zone of inhibition around the colony of antibiotic-producing bacteria indicated antibacterial activity (Fig. 1).

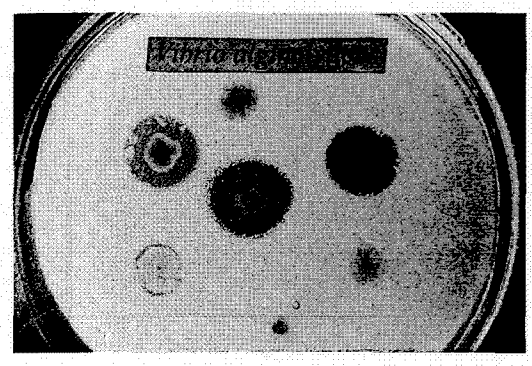

Fig. 1. Growth inhibition of *Vibrio alginolyticus* strain V07 displayed by marine bacteria

A strain (N06) of antibiotic-producing bacteria was cultured in Marine Broth 2216 (MB, Difco Laboratories) at 25°C for 3 days. The culture supernatant was prepared by centrifugation at 12,000 × *g* for 15 min and sterilized by filtration through a membrane filter (0.45 μm). The supernatant and MB were equally mixed and *V. penaeicida* KH-1 strain was inoculated in the mixture at doses of 10^3 and 10^7 CFU (colony forming unit) / m*l*. Heat-treated (70°C, 30 min) supernatant was served as a control. After shake culture at 25°C, viable bacterial counts were performed on MA plates.

Four strains, N01, N06, N18 and N29, giving broad spectrum inhibition for *Vibrio* species examined were selected for mix culture with 3 *Vibrio* species, *V. alginolyticus* V07, *V. parahaemolyticus* V13 and *V. harveyi* V22 to observe their interaction. Two-day cultures on MA of these bacteria were inoculated at each dose of 10^4 CFU/m*l* in basal medium containing yeast extract (5 g/*l*). The basal medium (pH 7.5) consisted of (g/*l*): tris-(hydroxymethyl)-aminomethane 6.1, NH_4Cl 1.0, $K_2HPO_4 \cdot 3H_2O$ 0.075, $FeSO_4 \cdot 7H_2O$ 0.028 and 1/2 strength artificial seawater (ASW). ASW was composed of (g/*l*): NaCl 23.4, $MgSO_4 \cdot 7H_2O$ 24.6, KCl 1.5, $CaCl_2 \cdot 2H_2O$ 2.9 and distilled water. Mono-species culture of each strain was served as a control. After 3-day shake incubation at 30°C, 0.1 m*l* of each sample was taken from the cultures, diluted and then spread onto MA (2% agar) and Thiosulphate Citrate Bile salt Sucrose Agar (TCBS, Difco Laboratories). TCBS was used for counting *Vibrio* cells. CFUs on these media were enumerated after incubating at 30°C for 2 days.

Identification of antibiotic-producing bacteria

Twenty seven strains of marine bacteria that inhibited the growth of *Vibrio* strains tested were subjected to taxonomic characterization. Unless otherwise stated, all cultures were incubated at 30°C. The characteristics tested included colony and cell morphology, Gram stain, pigment production, motility, flagellation, production of oxidase and catalase, O-F test, nitrate reduction, growth at 4°C and 40°C, citrate utilization and seawater requirement. Productions of lipase, amylase, gelatinase and deoxyribonuclease were examined. The ability in utilizing different organic compounds as a sole carbon source was determined on the basal medium agar containing 0.2% of the tested sugars or 0.1% of the other compounds (Krieg and Holt, 1984). The mol % guanine and cytosine (G + C) content of DNA was analyzed by HPLC method (Kumagai *et al.*, 1988). Identification was made by referring to Bergey's Manual of Systematic Bacteriology Vol. 1 (Krieg and Holt, 1984) and Bergey's Manual of Determinative Bacteriology 9th ed. (Holt *et al.*, 1994).

Results

Inhibition activity against Vibrio

Forty five strains of marine bacteria showing anti-swarming effects against swarming bacteria were tested for their potentiality in inhibiting 50 strains of isolated vibrios including *V. parahaemolyticus* (3 strains), *V. fluvialis* (5), *V. alginolyticus* (8), *V. harveyi* (10) and *Vibrio* spp. (24) by the double-layer plate method (Table 1). The test revealed that 27 isolated strains inhibited 32 to 94% of the *Vibrio* strains tested (Table 2). Among these, strain N06 was the most active strain inhibiting 94% of vibrios assayed. Clear zones of inhibition of this strain against vibrios were also wider as compared with the others. The strains of N01, N18, N25 and N29 displayed inhibitory activity against 90% of vibrios strains tested. In addition to isolated vibrios mentioned

Table 2. Inhibitory activity of 45 marine bacteria against 50 strains of *Vibrio* spp. tested by a double-layer plate method

Marine bacterial strain	*V. para-haemolyticus* (N = 3)*	*V. fluvialis* (N = 5)	*V. algino-lyticus* (N = 8)	*V. harveyi* (N = 10)	*Vibrio* spp. (N = 24)	Number of *Vibrio* strains inhibited	% *Vibrio* strain inhibited
N01	2**	5	5	10	23	45	90
N02	2	5	2	10	20	39	78
N03	0	0	0	0	0	0	0
N04	1	4	2	10	19	36	72
N05	2	4	5	10	22	43	86
N06	2	5	7	10	23	47	94
N07	0	0	0	0	0	0	0
N08	2	5	6	10	17	40	80
N09	1	4	0	7	14	26	52
N10	0	1	0	1	2	4	8
N11	2	4	5	10	19	40	80
N12	0	0	0	0	1	1	2
N13	0	0	0	0	2	2	4
N14	0	0	0	0	1	1	2
N15	1	4	0	9	15	29	58
N16	0	0	0	0	1	1	2
N17	0	1	0	6	9	16	32
N18	2	4	8	10	21	45	90
N19	2	5	7	10	20	44	88
N20	0	0	0	0	0	0	0
N21	0	0	0	0	0	0	0
N22	1	5	4	10	21	41	82
N23	1	5	6	10	16	38	76
N24	2	4	1	10	15	32	64
N25	2	5	7	10	21	45	90
N26	2	3	2	8	17	32	64
N27	1	3	4	9	21	38	76
N28	2	3	5	10	21	41	82
N29	2	4	7	10	22	45	90
N30	0	0	0	0	0	0	0
N31	0	0	0	0	0	0	0
N32	0	1	0	0	0	1	2
N33	1	3	1	3	14	22	44
N34	2	4	6	10	19	41	82
N35	0	0	0	0	0	0	0
N36	0	0	0	0	0	0	0
N37	0	3	1	5	15	24	48
N38	0	0	0	0	0	0	0
N39	2	5	4	10	21	42	84
N40	0	0	0	0	0	0	0
N41	2	5	4	10	22	43	86
N42	2	5	4	10	22	43	86
N43	0	0	0	0	0	0	0
N44	2	4	5	9	17	37	74
N45	0	0	0	0	0	0	0

* Number of strains tested, ** Number of strains inhibited.

above, these antibiotic-producing strains also exhibited inhibitory activity against 52 to 96% of 27 reference strains of *Vibrio* species (Table 3). Conversely, no *Vibrio* strains inhibited the growth of these *Vibrio*-inhibiting strains when some selected strains were examined, but there was cross inhibition among the

Table 3. Inhibitory activity of marine bacteria against 27 strains of reference *Vibrio* species tested by a double-layer plate method

Marine bacterial strain	% *Vibrio* strain inhibited	Marine bacterial strain	% *Vibrio* strain inhibited	Marine bacterial strain	% *Vibrio* strain inhibited
N01	92.6	N16	NT	N31	NT
N02	85.2	N17	59.3	N32	NT
N03	NT*	N18	88.9	N33	51.9
N04	88.9	N19	92.6	N34	96.3
N05	85.2	N20	NT	N35	NT
N06	96.3	N21	NT	N36	NT
N07	NT	N22	88.9	N37	55.6
N08	88.9	N23	92.6	N38	NT
N09	85.2	N24	88.9	N39	85.2
N10	NT	N25	88.9	N40	NT
N11	92.6	N26	77.8	N41	85.2
N12	NT	N27	81.5	N42	81.5
N13	NT	N28	81.5	N43	NT
N14	NT	N29	85.2	N44	74.1
N15	74.1	N30	NT	N45	NT

* not tested

Table 4. Growth inhibition of *V. penaeicida* KH-1 by culture supernatant of antibiotic-producing marine bacterium strain N06

Incubation time (h)	Initial dose: 10^7 CFU/ml		Initial dose: 10^3 CFU/ml	
	Experiment (CFU/ml)	Control (CFU/ml)	Experiment (CFU/ml)	Control (CFU/ml)
0	3.4×10^7	2.6×10^7	2.0×10^3	2.6×10^3
1	2.6×10^7	3.0×10^7	1.3×10^3	2.3×10^3
3	3.4×10^7	1.7×10^7	1.0×10^3	2.8×10^3
5	4.6×10^6	3.5×10^7	7.0×10^1	3.8×10^4
9	< 10	4.0×10^7	< 10	3.7×10^4
24	< 10	1.6×10^8	< 10	1.1×10^8

V. penaeicida was shake-cultured with the culture supernatant of strain N06 in MB at 25°C.

strains of antibiotic-producing strains.

The CFU of *V. penaeicida* KH-1 strain decreased after 5 h incubation in cell-free culture supernatant of strain N06 and were undetectable (< 10 CFU/ml) after 9 h incubation (Table 4). The same decreasing tendencies were observed in the incubations with initial doses of 10^3 and 10^7 CFU/ml. In contrast, the bacterial numbers reached 10^8 CFU/ml after 24 h incubation in heat-treated supernatant.

Interaction between each strain of antibiotic-producing N01, N06, N18, N29 and each species of *V. alginolyticus* V07, *V. parahaemolyticus* V13 and *V. harveyi* V22 is given in Table 5. Each mono-culture reached 10^8 to 10^9 CFU/ml after 3-day incubation. A slight decrease was noticed in the bacterial numbers of *V. alginolyticus* V07 and *V. harveyi* V22 in their mix-cultures with the antibiotic-producing bacteria but not in *V. parahaemolyticus* V13.

Identification of antibiotic-producing marine bacteria

Morphological and biochemical characteristics of the 27 strains of antibiotic-producing bacteria are shown in Table 6. The colonies of these bacteria were circular, convex and entire. They were Gram negative aerobes, short rods 1.0–1.5 × 2.0–2.5 μm in size and motile by means of a single polar flagellum. All required a seawater base for growth and did not produce acids from glucose but used glucose as a sole carbon source except

Table 5. Interaction between antibiotic-producing marine bacteria and *Vibrio* species in mix culture

Bacterial strain	Number of bacteria (log CFU/ml)	
	day 0	day 3
N01 : none	4.3 : 0	8.1 : 0
N06 : none	3.9 : 0	8.5 : 0
N18 : none	4.1 : 0	8.5 : 0
N29 : none	4.5 : 0	8.1 : 0
none : V07	0 : 4.3	0 : 8.1
N01 : V07	4.3 : 4.3	8.0 : 7.6
N06 : V07	3.9 : 4.3	8.3 : 7.7
N18 : V07	4.1 : 4.3	8.2 : 7.5
N29 : V07	4.5 : 4.3	8.1 : 7.6
N01 : none	4.3 : 0	8.8 : 0
N06 : none	4.3 : 0	8.5 : 0
N18 : none	4.4 : 0	8.9 : 0
N29 : none	4.2 : 0	8.8 : 0
none : V13	0 : 4.3	0 : 7.9
N01 : V13	4.3 : 4.3	8.1 : 8.0
N06 : V13	4.3 : 4.3	8.6 : 7.5
N18 : V13	4.4 : 4.3	8.1 : 8.0
N29 : V13	4.2 : 4.3	7.9 : 8.0
N01 : none	4.6 : 0	8.2 : 0
N06 : none	4.7 : 0	8.7 : 0
N18 : none	4.5 : 0	8.8 : 0
N29 : none	4.5 : 0	8.7 : 0
none : V22	0 : 4.4	0 : 8.7
N01 : V22	4.6 : 4.4	8.8 : 8.4
N06 : V22	4.5 : 4.4	8.9 : 7.8
N18 : V22	4.5 : 4.4	8.5 : 8.1
N29 : V22	4.5 : 4.4	7.9 : 8.2

N01, N06, N18, N29: antibiotic-producing bacteria, V07: *V. alginolyticus*, V13: *V. parahaemolyticus*, V22: *V. harveyi*.

the strains of group 4. The G + C content of DNA was 42.4 to 45.3% mol. Based on these characteristics, they were tentatively identified to the genus *Alteromonas*. Further characteristics could not identify them to the species level, and they were divided into 4 groups according to differentiation of some characteristics. Group 1 included 19 strains: N01, N05, N06, N08, N11, N18, N19, N22, N25, N26, N27, N28, N29, N33, N37, N39, N41, N42 and N44. Group 2 included 3 strains: N23, N24 and N34. The strains in group 1 produced yellow or yellowish orange pigment while those in group 2 produced orange pigment on MA. Group 1 was distinguished from group 2 by their inabilities to produce oxidase as well as their abilities to utilize sucrose. Group 3 included 3 strains: N02, N15 and N17, and group 4 included 2 strains: N04 and N09. These two groups produced no pigments on MA. Group 3 differed from group 4 in their abilities to utilize D-mannose, maltose, N-acetylglucosamine, succinate, fumarate, D-glucose and D-trehalose.

Discussion

Up to the present, more than 1,000 antibiotics have been discovered. Most of these have been isolated from fungi and bacteria which are terrestrial in origin. Only a few organisms in marine environment are known to produce antibiotics (Gauthier and Flatau, 1976; Gauthier, 1977; Imada *et al.*, 1985; Dopazo *et al.*, 1988).

In the present study, screening of antibiotic-producing marine bacteria was made by inhibition of bacterial swarming as an indicator. Such screening method gave a good result since 27 out of 45 strains isolated inhibited 32 to 94% of vibrios tested. Among these 27 strains, particularly N01, N06, N18, N25, and N29 exhibited higher inhibition against vibrios, however there seemed to be no correlation between their activities and their sources of isolation. These strains also had inhibitory activities widely against reference strains of 27 *Vibrio* species including *V. penaeicida* (Ishimaru *et al.*, 1995), the pathogen of vibriosis of cultured kuruma prawn *Penaeus japonicus* in Japan. This inhibition acts bactericidally and the antibiotic substance(s) was found to be extracellularly produced and heat-labile (70°C, 30 min) (Table 4). However, the results of mix culture in the liquid medium were not so remarkable as those obtained on solid medium or in the experiment using culture supernatant and *V. penaeicida*, in which bacteria were grown for 3 days prior to the inhibition assay, suggesting that plenty of antibiotic are required at the initiation of mix culture for the evident inhibition. This will be a problem to be considered in disease control using these *Vibrio*-inhibiting marine bacteria. Similar results were reported by Dopazo *et al.* (1988) that *Aeromonas hydrophila* and *V. anguillarum* showed resistance in a liquid medium to some strains of the marine bacteria that displayed inhibitory activity on solid medium. But in their test in the liquid medium, *A. salmonicida* was rapidly inhibited by the marine bacteria being reduced in viable count by more than 4 logs after 1 h.

In the present study, all 27 isolates of *Vibrio*-inhibiting marine bacteria were identified to *Alteromonas*-like organisms but were unidentified to the species level because they failed to match exactly with any established

Table 6. Characteristics of marine bacteria producing antibiotic against various species of *Vibrio*

Characteristic	Group 1 19 strains	Group 2 3 strains	Group 3 3 strains	Group 4 2 strains
Gram stain	–	–	–	–
Cell shape	short rod	short rod	short rod	short rod
Cell size (μm)	1.0–1.5 × 2.0–2.5	1.0–1.5 × 2.0–2.5	1.0–1.5 × 2.0–2.5	1.0–1.5 × 2.0–2.5
Pigmentation	+	+	–	–
Motility	+	+	+	+
Flagellation	single polar	single polar	single polar	single polar
Oxidase	–	+	–	–
Catalase	+	+	+	+
OF test	–	–	–	–
Nitrate reduction	–	–	+	+
Growth at:				
4°C	–	–	–	–
40	+w	+	+	+
Seawater requirement	+	+	+	+
Production of:				
Amylase	+	+	+	+
Gelatinase	+ (17)*	+	+	+
Lipase	+	+	+	+
Alginase	–	–	–	–
Chitinase	–	–	–	–
Deoxyribonuclease	+	+	+	+
Citrate (Simmons)	+	+	–	–
Utilization of:				
D-Mannose	+	+	+	–
D-Galactose	–	–	–	–
D-Fructose	–	–	–	–
Sucrose	+	–	–	–
Maltose	+	+	+	–
Cellobiose	–	–	–	–
N-Acetylglucosamine	+	+	+	–
Succinate	+w (18)	+w	+w	–
Fumarate	+w	+w	+w	–
Erythritol	–	–	–	–
D-Mannitol	–	–	–	–
Glycerol	–	–	–	–
DL-Malate	–	–	–	–
α-Ketoglutarate	–	–	–	–
D-Glucose	+	+	+	–
D-Trehalose	+	+	+	–
Mol% G + C of DNA	42.4–45.3	43.4–43.8	42.6–43.3	42.7–44.8

* number of strains

Group 1: N01, N05, N06, N08, N11, N18, N19, N22, N25, N26, N27, N28, N29, N33, N37, N39, N41, N42 and N44; Group 2: N23, N24 and N34 ; Group 3: N02, N15 and N17; Group 4: N04 and N09

species of the genus *Alteromonas*. In the Manual of Systematic Bacteriology Vol. 1 (Krieg and Holt, 1984), the genus *Alteromonas* was characterized into 11 species and 4 of these, *A. rubra*, *A. luteoviolacea*, *A. citrea* and *A. aurantia*, produce antibiotic substances of high molecular weight. All of the four species have been isolated from the Mediterranean Sea. Bacteria of this genus were also isolated from intertidal green and brown algae in the north-west of Spain and displayed inhibitory activity against bacterial fish pathogens belonging to the genera *Vibrio*, *Aeromonas*, *Pasteurella*, *Edwardsiella*, and *Yersinia* (Dopazo *et al.*, 1988).

The isolation of *Alteromonas*-like strains from the larvae of black tiger shrimp in this study indicates that

such marine bacteria may play an important role in the natural resistance of shrimp to bacterial pathogens especially of the genus *Vibrio*. Further studies on the suitable culture condition to elicit higher production of antibiotic substance as well as purification and characterization of their products are necessary. A preliminary experiment indicates that the antibiotic substance of the present *Alteromonas*-like bacterium (strain N06) is a thermolabile (70°C, 30 min) protein with high molecular weight (> 50,000 Da) (data not shown). Simultaneously, efficacy of these marine bacteria on the reduction of bacterial contamination of brine shrimp and larval black tiger shrimp needs to be evaluated, in order to use these marine bacteria for controlling epizootics in shrimp seed production.

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References

Dopazo, C. P., M. L. Lemos, C. Lodeiros, J. Bolinches, J. L. Barja and A. L. Toranzo (1988): Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. *J. Appl. Bact.*, **65**, 97–101.

Flegel, T. W., D. F. Fegan, S. Kongsom, S. Vuthikomudomkit, S. Sriurairatana, S. Boonyaratpalin, C. Chantanachookhin, J. E. Vickers and O. D. Macdonald (1992): Occurrence, diagnosis and treatment of shrimp diseases in Thailand. In "Diseases of cultured penaeid shrimp in Asia and the United States" (ed. by W. Fulks and K. L. Main), Oceanic Institute, Honolulu, pp. 57–112.

Gauthier, M. J. (1977): *Alteromonas citrea*, a new Gram-negative, yellow-pigmented species from seawater. *Int. J. Syst. Bacteriol.*, **27**, 349–354.

Gauthier, M. J. and G. N. Flatau (1976): Antibacterial activity of marine violet-pigmented *Alteromonas* with special reference to the production of brominated compounds. *Can. J. Microbiol.*, **22**, 1612–1619.

Holt, J. G., N. R. Krieg, P. H. A. Sneath, J. T. Staley and S. T. Williams (1994): Bergey's manual of determinative bacteriology, 9th ed. Williams and Wilkins, Baltimore, USA, 787 p.

Imada, C., U. Simidu and N. Taga (1985): Isolation and characterization of marine bacteria producing alkaline protease inhibitor. *Bull. Japan. Soc. Sci. Fish.*, **51**, 799–803.

Ishimaru, K., M. Akagawa-Matsushita and K. Muroga (1995): *Vibrio penaeicida* sp. nov., a pathogen of kuruma prawns (*Penaeus japonicus*). *Int. J. Syst. Bacteriol.* **45**, 134–138.

Krieg, N. R. and J. G. Holt (1984): Bergey's manual of systematic bacteriology, Vol. 1. Williams and Wilkins, Baltimore, USA, 964 p.

Kumagai, M., M. Fujimoto and A. Kuninaka (1988): Determination of the base composition of DNA by high performance liquid chromatography of its nuclease P1 hydrolysate. *Nucleic Acid Research*, Symposium Series No. 19, 65–68.

Muroga, K., M. Higashi and H. Keitoku (1987): The isolation of intestinal microflora of farmed red seabream (*Pagrus major*) and black seabream (*Acanthopagrus schlegeli*) at their larval and juvenile stages. *Aquaculture*, **65**, 79–88.

Ruangpan, L. (1981): Diseases and parasites of *Penaeus monodon* Fabricius. Technical Paper 2/198. Coastal Aquaculture Res. Sub-Div., Brackishwater Fish. Div., Dept. Fish. Thailand, 7 p.

Ruangpan, L. (1985): Protozoa which cause problems in shrimp culture (*Penaeus monodon* and *P. merguiensis*). Technical Paper 8/85. Coastal Aquaculture Res. Sub-Div., Brackishwater Fish. Div., Dept. Fish. Thailand, 15 p.

Sritongsuk, C. and S. Tookvinas (1991): Standard of shrimp farming quality. *Thai Fish. Gazette*, **44**, 19–21.

Tanasomwang, V. and L. Ruangpan (1995): Bacterial flora of larvae of the black tiger shrimp, *Penaeus monodon*. In "Diseases in Asian aquaculture II" (ed. by M. Shariff, J. R. Arthur and R. P. Subasinghe), Fish Health Section, Asian Fisheries Society, Manila. pp. 151–162.

Tanasomwang, V. and T. Supaiwan (1996): Susceptibility of bacteria isolated from *Penaeus monodon* larvae, ambient water and feeds to 9 antimicrobial agents. Technical Paper No. 12/19966. Samutsakhon Coastal Aquaculture Development Center, Coastal Aquaculture Div., Dept. Fish. Thailand, 13 p.

Tanasomwang, V. and T. Supaiwan (1997): Efficacy of formalin, benzalkonium chloride and povidone iodine in inhibiting *Vibrio* isolated from larval black tiger shrimp (*Penaeus monodon*), rearing water and *Artemia salina*. Technical Paper No. 18/1997. Samutsakhon Coastal Aquaculture Development Center, Coastal Aquaculture Div., Dept. Fish. Thailand, 17 p.

Tansutapanich, A., N. Phuwapanish and T. Sanggontanagit (1990): Improving of shrimp farm environment. *Thai Fish. Gazette*, **43**, 365–369.