アサヒガニ幼生飼育水槽から分離した優勢菌相の性状

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Characteristics of Dominant Microflora in Aquaculture Tanks of Juvenile Red Frog Crab, *Ranina ranina*

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Abstract

Bacterial counts and dominant microflora in aquaculture tanks of juvenile red frog crabs were compared in various culture conditions. Survival rates of juvenile crabs decreased drastically after zoea 5 stage in 30 *l* or 500 l culture tanks with 3 or 9 turns per 15 h of seawater exchange. In general, 10^4 to 10^5 cfu/m*l* of viable bacterial counts in seawater of culture tanks were obtained on Z-CII agar plates. Swarming colonies were commonly found in about one tenth level of total colony counts on Z-CII plates. Dominant strains forming whitish gray smooth or swarming colonies on Z-CII plates were found to be *Vibrio* spp. and especially swarming strains were identified as *V. alginolyticus* according to their bacteriological characterization and phage sensitivity.

The red frog crabs, *Ranina ranina* are widely distributed from Indian Ocean to Pacific Ocean. In Japan, they are important for local fisheries and one of the favorite crabs because of its good taste. In general, crabs are nocturnal and stay in sand during daytime. They cover their bodies with sand and their eyes and mouth parts poke out of the sand and they become active at night.[1] Since the mortality rate of the red frog crabs during the early juvenile stages was very high, it has been unsuccessful to attempt to culture the red frog crabs in the aquaculture tanks. The possible causative factors that affect mass mortality of the juvenile crabs are considered to be physical, chemical, and microbiological ones. In this study, we examined changes of dominant microflora in seawater of the aquaculture tanks during early juvenile stages of the red frog crabs in an attempt to reveal the causative agents for their mass mortality. Major infectious diseases for marine crustaceans are reported to be related to *Vibrio* species including *V. anguillarum, V. harveyi, V. penaeicida, V. alginolyticus,* and *V. parahaemolyticus.*[2-6] *Vibrio* species are ubiquitously distributed in estuarine and coastal areas in which marine animal population is very high.[7-10] *Vibrio* species were therefore paid more attention than other bacteria in this paper.

Materials and Methods

Culture Conditions of Juvenile Red Frog Crabs

The breeders of red frog crabs with eggs were caught at coastal areas of Kagoshima Prefecture and transported to Shibushi branch of Japan Sea-Farming Association. They were cultured in a seawater tank (1.5 m^3) containing sand on the bottom as with 10 times per day water exchange and fed with mussel meat until they spawned. Average fecundity was 105,000 per a female. Juvenile crabs were cultured in 30 *l* or 500 *l* polycarbonate tanks with seawater exchanged at 3, 5, and 9 turns per 15 h and fed with artemia once or twice per day. About

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300 and 5,000 juveniles were placed in 30 *l* and 500 *l* tanks, respectively. In the case of 10 *l* flat waterbaths, about 100 juveniles were kept in a waterbath and they were transferred from the waterbath to another one with seawater supplied freshly every day by using a 10 m*l* glass pipette.

Viable Count and Isolation of Bacteria

Seawater samples were aspetically collected from different cultural tanks for the juvenile crabs using sterile 300 m*l* capacity plastic bottles. Each seawater sample was serially diluted at one-tenth with sterile normal saline solution. An aliquot of appropriate dilution (0.1 m*l*) was spread on a Z-CII agar plate (polypeptone 5 g, yeast extract 1 g, agar 15 g, and artificial seawater 1 *l*, pH 7.6) and a BTB Teepol agar plate (beef extract 3 g, peptone 10 g, sucrose 10 g, Teepol 2 m*l*, NaCl 30 g, BTB 0.08 g, agar 15 g, and distilled water *1 l*, pH 7.8). The plates were incubated at 25 ℃ for 3-5 days and bacterial colonies were counted. Bacterial colonies were picked up at random from representative plates and inoculated streakingly on Z-CII agar plates to obtain purified colonies. The bacterial strains were incubated on Z-CII agar slants at 25 ℃ for 1 day and then kept in a cold room (10 ℃).

Characterization and Identification of the Isolates

Colony color and swarming were observed on Z-CII agar medium after incubation at 25 ℃ for 1-2 days. Motility of test strains was examined by hanging drop method. Gram stain was carried out according to the standard bacteriological method.[11] Fermentation of glucose or sucrose was determined after 7 days of incubation in Hugh and Leifson medium containing 1% glucose or 1% sucrose. Oxidase activity was determined by use of cytochrome oxidase paper (Nissui). Catalase activity was determined by bubble formation as bacterial cells were transferred into a test tube containing 3% hydrogen peroxide solution. Indole production, Voges-Proskauer test (VP), methyl red test (MR), lysine decarboxylase, or arginine dihydrolase were examined according to the standard bacteriological methods.[11] Hydrolysis of casein or starch was detected from lytic zones around colonies grown on Z-CII agar plates added with skim milk (2.0%) or starch (0.5%), respectively. Growth at different NaCl concentrations was determined after 7 days incubation in liquid media containing 1% polypeptone and different concentrations (0%, 7%, 10%) of NaCl.

Phage Sensitivity of Swarming Bacteria Strains

The representative swarming bacteria strains from each seawater sample were tested for phage sensitivity using four types of phage lysates which had been isolated in 1996. These phage lysates were obtained from the bacterial culture added with seawater samples of the culture tanks and purified by incubation with each host bacterium isolated in 1996 and had been maintained. Phage sensitivity of swarming bacteria was examined by the spot test. A small drop of each phage lysate solution was placed by using a micropipette on double layer agar plates containing bacterial cells grown in Z-CII broth cultured at 25 ℃ for 1 day. After incubation, the appearance of plaques was observed on the double layer agar plates.

Results

Survival Rate of Crab Juveniles

Survival rates of crab juveniles in aquaculture tanks are shown in Table 1. In 500 *l* tanks survival rates of juveniles were relatively high until zoea 4 stage but after that it decreased drastically. In the culture tanks with water exchange rate of 9 turns per 15 h, the survival rates were somewhat higher than those of the tanks with 3 turns per 15 h but at zoea 7 stage alive juveniles were very few in both tanks. In 10 *l* flat waterbath 100 crab juveniles were transferred using a glass pipette into the different one with seawater exchanged freshly every day and survival rates of juveniles were sufficiently higher (10 to 20% constantly) than those in 30 *l* or 500 *l* of culture tanks.

Bacterial Counts in Seawater of Juvenile Culture Tanks

Viable bacterial counts of seawater samples from

various culture tanks at an early stage (zoea 2 or 3) of juvenile crabs are shown in Figs. 1 and 2. In general, 10^4 to 10^5 cfu/m*l* were obtained on Z-CII plates as viable counts in water samples. Viable bacterial counts in tanks with 9 turns per 15 h of water exchange (S-7 in Fig. 1) were slightly lower than those in tanks with 3 turns per 15 h (S-8 in Fig.1). In 10 *l* flat waterbaths viable counts of culture water increased 5 to 10 times during 24 h keeping of about 100 juveniles (S-7 and S-8 in Fig. 2). On Z-CII plates swarming colonies were commonly found in one tenth level of total viable counts for almost all samples of tank waters. On the other hand yellow colonies occurred much greater than green colonies on BTB Teepol plates. In general. total bacterial counts on BTB Teepol plates were about 35% of total counts on Z-CII plates.

Characterization of Swarming Bacteria

Dominant strains isolated on Z-CII plates, which formed whitish gray smooth colonies or swarming colonies, were identified as *Vibrio* spp. according to the bacterial identification scheme proposed by Simidu.[12] Main characteristics of swarming bacteria commonly found in all water samples are demonstrated in Table 2 and Fig. 3. Most of swarming bacteria exhibited yellow colony formation on BTB Teepol agar, oxidase-positive, catalase-positive, acid

Table 1 Survival rates of juvenile red frog crabs in 30 or 500 *l* culture tanks

Culture conditions (water exchange rate)	Volume of water	No. of crabs	Survival rates at zoea stages (%)						
			Z_2	Z_3	Z_4	Z_5	Z_6	Z_7	Megalopa
9 turns/15h (transparent tank)*1	500 *l*	5,000	97	90	81	52	20	5	0
3 turns/15h (transparent tank)	500 *l*	5,000	99	90	78	44	11	4	0
3 turns/15h (black tank)	500 *l*	5,000	95	87	71	29	3	0	0
5 turns/15h (streptomycin, not added)	30 *l*	300	97	86	74	53	31	16	0
5 turns/15h (streptomycin 50 ppm)*2	30 *l*	300	99	93	81	62	41	23	0

*1 9 turns per 15 h of water exchange in 500 *l* transparent polycarbonate tank.
*2 5 turns per 15 h of water exchange with seawater added 50 ppm of streptomycin in 30 *l* tank.

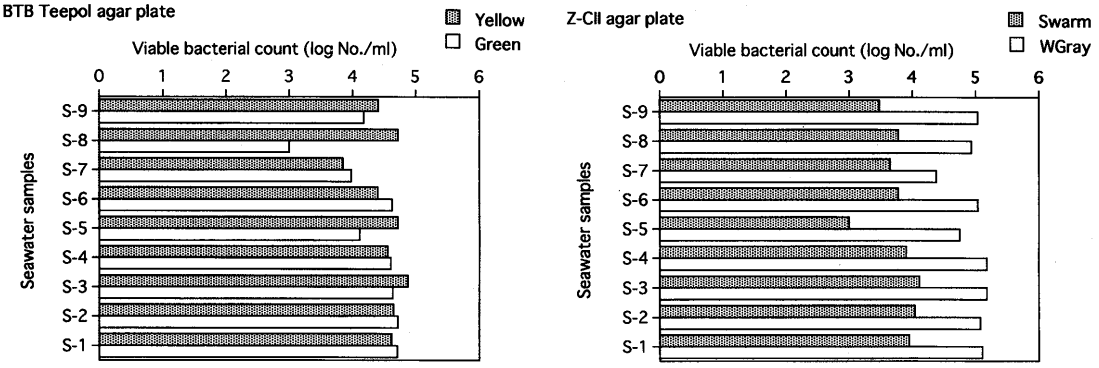

Fig. 1 Viable bacterial counts on Z-CII and BTB Teepol agar plates in seawater samples obtained from various culture tanks with juvenile red frog crabs in 1998.
Juvenile red frog crabs after hatching were kept for two days in 30 *l* or 500 *l* polycarbonate tanks with feeding of artemia. "Yellow" and "Green" indicate yellow and green colonies on BTB Teepol agar plates, respectively. "Swarm" and "WGray" indicate swarming and whitish gray colonies on Z-CII agar plates, respectively.
S-1, a seawater sample from 30 *l* culture tank with 5 turns of water change per 2.5 h and twice feeding a day; S-2, 30 *l* culture tank with 3 turns of water change per 15 h and twice feeding a day; S-3, 30 *l* culture tanks with 3 turns of water change per 15 h, addition of commercial "Marine Growth" enrichments, and twice feeding a day; S-4, 30 *l* culture tank with 3 turns of water change per 15 h and once feeding a day; S-5, 500 *l* culture tank with 50% of water exchange a day; S-6, 500 *l* culture tank with 50% of water exchange a day; S-7, 500 *l* culture tank with 9 turns of water change per 15 h and once feeding a day; S-8, 500 *l* culture tank with 3 turns of water change per 15 h and once feeding a day; S-9, 500 *l* culture tank with 3 turns of water change per 15 h, addition of commercial "Marine Growth" enrichments, and once feeding a day.

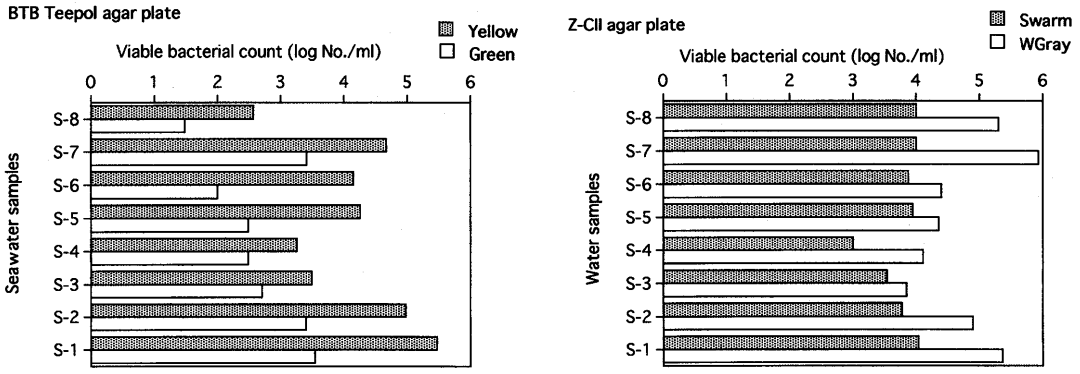

Fig. 2 Viable bacterial counts on Z-CII and BTB Teepol agar plates in seawater samples obtained from various culture tanks with juvenile red frog crabs in 1999.
Juvenile red frog crabs after hatching were kept for two days in 10 *l* flat waterbath, 30 *l* or 500 *l* polycarbonate tanks. S-1, a seawater sample from surface layer of 500 *l* culture tank with 9 turns of water change per 15 h and once feeding a day; S-2, a seawater sample of the bottom of 500 *l* culture tank with 9 turns of water change per 15 h and once feeding a day; S-3, a seawater sample from surface layer of 30 *l* culture tanks with 9 turns of water change per 15 h and twice feeding a day; S-4, a seawater sample from the bottom of 30 *l* culture tank with 9 turns of water change per 15 h and twice feeding a day; S-5, a seawater sample from surface layer of 30 *l* culture tanks with 9 turns of water change per 15 h and once feeding a day; S-6, a seawater sample from the bottom of 30 *l* culture tank with 9 turns of water change per 15 h and once feeding a day; S-7, 10 *l* flat waterbath after one day without water exchange and with once feeding; S-8, 10 *l* flat waterbath immediately after transfer of juvenile red frog crabs to it.

Table 2 Main characteristics of the representative strains of swarming bacteria

Characters	1S1-1*[1]	1S1-2	2S1-1	2S1-3	3S3-1	3C2-1	4S1-2	4S2-7	S98-A
BTB Teepol	Y[2]	Y	Y	Y	Y	Y	Y	Y	Y
Swarm	S	S	S	S	S	S	S	S	S
Gram	−	−	−	−	−	−	−	−	−
Cell form	R	R	R	R	R	R	R	R	R
Motility	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
VP test	+	+	−	+	+	−	−	+	+
MR test	−	−	−	−	−	−	−	−	−
Indole production	+	+	−	−	+	+	−	+	+
Glu. fermentation	F	F	F	F	F	F	F	F	F
Suc. fermentation	F	F	F	F	F	F	F	F	F
Arg. dihydrolase	+	−	−	−	−	−	−	−	−
Lys. decarboxylase	−	+	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+
Growth in NaCl (0%)	−	−	−	−	−	−	−	−	−
NaCl (7%)	−	+	+	+	+	+	+	+	+
NaCl (10%)	−	+	+	+	+	+	+	+	+

*[1] Representative strains of swarming bacteria isolated from seawater samples in 1998.
*[2] Abbreviation: Y, yellow; S, swarm; R, rod; F, fermentative.

production from glucose and sucrose in Hugh and Leifson medium, VP test-positive, arginine dihydrolase-negative, lysine decarboxylase-positive, and growth in 10% NaCl peptone broth. Swarming strains isolated in 1998 were found to contain the strains sensitive to bacteriophages isolated from culture waters in 1996 as shown in Table 3. Especially, number of strains sensitive to bacteriophages ϕ 96III and ϕ 96IX was much more than that to ϕ 96II and ϕ 96IV.

Discussion

This study was undertaken to determine causative agents affecting mass mortality of juvenile red frog crabs and to help to develop a successful culture system. However, we could not make sure of the causative agents in this study. In 30 or 500 *l* culture tanks, filtered sea water was exchanged through a T-shaped plastic pipe with nylon mesh 3 to 9 turns per 2.5 or 15 h. On the other hand, individual juveniles were transferred every days by use of a glass pipette from one flat waterbath with seawater kept for one day to another one with seawater freshly exchanged. Bacterial counts in culture seawater containing about 100 juveniles increased 5 to 10 times when the water was not changed for one day.

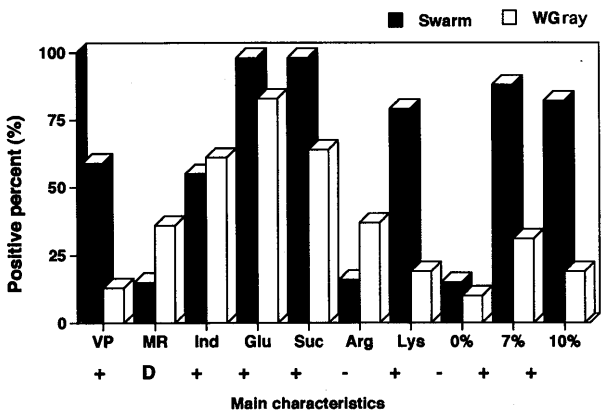

Fig. 3 Main characteristics of dominant strains isolated from seawater of culture tanks in 1998.
"Swarm" and "WGray" indicate bacterial strains which form swarming and whitish gray colonies on Z-CII agar plates, respectively. The upper line of horizontal indicate bacterial characteristics as shown in Table 2 and the lower line indicate the characteristics of *V. alginolyticus* according to Bergey's manual of systematic bacteriology.[9]

However, the survival rates of juveniles (10-20% at zoea 7 stage, data not shown) in flat waterbaths were constantly higher than those in 30 *l* or 500 *l* culture tanks. These observations suggest that mass mortality of crab juveniles may be caused by combined agents including physical, chemical and bacterial stresses.

Dominant bacterial flora in the culture waters was found to be composed of various *Vibrio* spp. including swarming bacteria. Since swarming bacteria were isolated commonly in all water samples as one of dominant bacteria, we attempted to characterize and identify swarming strains. They possessed specific features such as acid production from sucrose, arginine dihydrolase negative, lysine decarboxylase positive, and growth response in 10% NaCl peptone broth. According to these characteristics along with their phage sensitivities, the swarming strains isolated from the culture waters were identified as *Vibrio alginolyticus*. *V. alginolyticus was* reported to play a significant role in disease causing the high mortality of kuruma prawns. It remains to be clarified whether *V. alginolyticus* affects mass mortality of red frog crabs or not.

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Table 3 Phage sensitivity of the representative strains of swarming bacteria

Bacteria strains*1	Phage lysates tested*2			
	ϕ 96II	ϕ 96III	ϕ 96IV	ϕ 96IX
1S1-1	−	−	−	−
1S1-2	−	−	−	−
1S1-3	−	+t*3	−	−
1S2-1	−	+t	−	−
1S2-2	−	+t	−	−
1S2-3	−	−	−	−
1S3-1	−	−	−	−
1S3-2	−	+t	−	−
1S3-3	−	−	−	−
1S4-1	−	−	−	−
1S4-2	−	++c	+t	+t
1S4-3	−	+t	−	−
1S9-1	−	+t	−	−
1S9-2	−	+t	−	−
1S9-3	−	+t	−	−
3S4-1	−	−	−	−
3C1-1	−	−	−	−
3C1-2	−	−	−	−
3C1-3	−	−	−	−
3C2-1	−	+v	−	+v
3C2-2	−	++t	−	−
3C2-3	−	+t	+v	+t
3C3-1	−	+t	−	+v
3C3-2	−	+t	−	+t
3C3-3	−	+t	−	+t
96II	++t	−	−	−
96III	−	++t	−	−
96IV	−	++t	++t	++t
96IX	−	−	++c	++c

*1 Bacterial strains isolated from culture seawater in 1998.
*2 Phage lysates isolated from culture seawater in 1996.
*3 +t and ++t, turbid plaque; ++c, clear plaque; +v, very turbid plaque.

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