Microflora in the Gut of Japanese Coastal Crustacea

Haruo Sugita,^{*1} Ryutaro Ueda,^{*1} Leslie R. Berger,^{*2}
and Yoshiaki Deguchi^{*1}

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Bacterial populations in gut contents and gills of seven marine crustacea were investigated qualitatively and quantitatively using nine different agar media. Total viable counts of the gut contents ranged from 10^5 to 10^9 cells g^{-1} and, of the gills, from 10^6 to 10^7 cells g^{-1} . Three types of anaerobes, the Gram-positive coccus, Gram-positive rod and Gram-negative rod, were detected in 16.3%, 9.3% and 2.3%, respectively, of the crustacean samples examined. Although *Pseudomonas* and 6 of the 20 subgroups of the genus *Vibrio* were distributed widely among the crustacea, other bacterial components were also present in significant number in the selected crustacean species, individual specimens, or specimen tissues. Furthermore, cardinal growth temperatures of the bacterial isolates obtained in this study suggest the surrounding water temperature to be a major factor influencing apparent seasonal shifts in the gut microflora.

Aquatic animals always take a large number of bacteria into their gut from water, sediment and/or food. Most of these bacteria are temporary residents, frequently because of incompatible physical and chemical conditions, lethal interactions between bacteria, and/or immune responses in the gut of the host animals.¹⁾ Certain bacteria are present in the gut for a relatively long term and they form the gut microflora specific to the host animals.

Although much information has been recently obtained with respect to the microflora of aquatic vertebrates, including fish, frogs and turtles, some knowledge is also available about that of crustacea.¹⁾ The gut microflora of copepoda *Acartia tonsa*,²⁾ amphipod *Boeckosmus affinis*,³⁾ antarctic krill *Euphausia superba*,⁴⁾ scampi *Nephrops norvegicus*,⁵⁾ freshwater shrimp *Palaemon paucidens*,⁶⁾ Japanese prawn *Penaeus japonicus*,⁷⁾ giant prawn *Machrobrachium rosenbergii*,⁸⁾ blue crab *Callinectes sapidus*⁹⁾ and Majidae crab *Chionoecetes opilio*¹⁰⁾ has been studied. These reported all showed that the aerobic heterotrophs, including *Vibrio*, *Pseudomonas* and coryneforms, were predominant in the gut of crustacea. Recently, obligately anaerobic bacteria have been isolated as the dominant micro-organisms from the gut contents of aquatic vertebrates.¹¹⁻¹⁵⁾ However, few publications have dealt with anaerobes in the gut of

crustacea. In this paper we describe the results of qualitative and quantitative investigations of viable heterotrophic bacteria, including anaerobes, in the gut of coastal crustacea.

Materials and Methods

Animals

All animals examined were caught by gill nets in the period from October 1984 to August 1985 at Shimoda, Shizuoka Prefecture, Japan. Water temperature during the period of collection ranged from 13.8°C in March to 26.0°C in August. The animals included 5 specimens of Japanese spiny lobster *Panulirus japonicus* (45.5 to 54.6 g in body weight), and 6 species of coastal crabs, including 6 specimens of *Atergatis floridis* (8.0 to 27.8 g), 5 of *Schizophrys aspera* (11.7 to 17.1 g), 4 of *Tiarinia cornigera* (9.3 to 12.5 g), 5 of *Pachygrapsus crassipes* (5.6 to 16.3 g), 5 of *Thalamita prymna* (29.0 to 56.0 g) and 8 of *Plagusia dentipes* (23.5 to 40.2 g).

Media

Nine different agar media were used:

PYBG, 1/20PYBG, N-PYBG and modified PEA[MPEA] agar media were already reported.¹⁶⁾ TCBS and BTB-Teepol agar media were commercially supplied from Eiken Co. (Tokyo).

MAGPC medium,¹⁷⁾ a selective medium for

^{*1} Department of Fisheries, Nihon University, Shimouma, Setagaya, Tokyo 154, Japan (杉田治男, 上田龍太郎, 出口吉昭: 日本大学農獣医学部水産学科).

^{*2} Department of Microbiology, University of Hawaii, Honolulu, Hawaii, 96822, USA.

marine anaerobic Gram-positive cocci, containing (in g per 1000 ml of distilled water): Proteose peptone No. 3 (Difco), 10; Trypticase peptone (BBL), 3; Phytone peptone (BBL), 3; Lab-lemco powder (Oxoid), 2.4; liver extract (Kyokuto), 5; Bacto-yeast extract (Difco), 5; soluble starch, 0.5; glucose, 10; K_2HPO_4 , 1; KH_2PO_4 , 1; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 15; $MnSO_4$, 0.007; L-cysteine hydrochloride, 0.5; $FeSO_4 \cdot 7H_2O$, 0.2; Polysorbate 80, 1; β -phenylethyl alcohol, 3; Bacto-agar, 15; adjusted to pH 7.2.

Modified DHL[MDHL] medium, which is Desoxycholate medium (Eiken) supplemented with 2% (w/v) NaCl.

Modified FM-CW[MFM-CW] medium, which is FM-CW medium (Eiken) supplemented with 5% (v/v) horse blood and 1.5% (w/v) of NaCl.

Bacteriological Sampling

Gut contents and gills were aseptically removed, liquified, quantitatively diluted with the seawater diluent and then inoculated onto the nine different agar media described above. The PYBG, 1/20 PYBG, TCBS, BTB, MDHL, and MPEA agars were incubated aerobically and the PYBG, N-PYBG, MFM-CW, MPEA and MAGPC agars were incubated anaerobically, at 20°C for 12 to 14 days. Anaerobiosis was established in the anaerobic jar using GasPak anaerobic system (BBL).

After incubation, the bacterial colonies were divided into types according to colonial characteristics, that is, shape, size, elevation, structure, surface, edge, color and opacity, and the number of colonies of each recognizable type was counted in all the agar media. Three representatives of each colony type were streaked onto PYBG or 1/20PYBG agars, and purified by a second plating into the same medium.

Identification of Bacteria

The bacteria isolated aerobically, were identified to genera according to a modification¹⁸⁾ of the scheme of Shewan *et al.*,¹⁹⁾ and the manuals of Cowan¹⁹⁾ and Krieg and Holt.²⁰⁾ Vibrionaceae were further classified by the ability to form pigment and swarm on PYBG agar; to grow at 37°C; produce indole and acetylmethylcarbinol (VP reaction); reduce nitrate; produce any acid from sucrose, arabinose and inositol; and react on TCBS agar. Obligate and facultative anaerobes isolated under anaerobic conditions were classified on the basis of Gram reaction, cellular morphology, spore formation and the ability to grow

aerobically.

In suitable dilutions of each of the culture plates, the number of colonies of each bacterial group was counted and calculated into a count per 1 g of gut or gill material (wet weight). The maximum count for each bacterial group among the nine media was regarded as an accurate viable counts of the corresponding bacterial group. Total viable count (TVC) in each sample was obtained by summation of the viable counts of each bacterial group.

Growth Response of Aerobic Isolates at Various Temperatures

The bacterial strains isolated aerobically from *Plagusia* and *Atergatis* crabs were inoculated onto PYBG agars and incubated aerobically at various temperatures as follows: 5 and 10°C, 20 days; 20, 25 and 30°C, 7 days; 37 and 42°C, 2 days.

Results

Colony Counts of Bacteria

The number of colonies that formed on each type of agar medium varied from one sample to the next as follow: PYBG, 10^5 to 10^8 cells g^{-1} under both aerobic and anaerobic conditions; 1/20PYBG, 10^6 to 10^8 cells g^{-1} ; TCBS, 10^4 to 10^8 cells g^{-1} ; BTB, 10^4 to 10^8 cells g^{-1} ; MDHL, 10^4 to 10^8 cells g^{-1} ; MPEA, 10^5 to 10^8 cells g^{-1} under aerobic conditions, 10^4 to 10^7 cells g^{-1} under anaerobic conditions; N-PYBG, 10^4 to 10^8 cells g^{-1} ; MFM-CW, $<2 \times 10^2$ to 10^7 cells g^{-1} ; MAGPC, 10^4 to 10^7 cells g^{-1} .

Bacteria Isolated

A total of 1564 strains of aerobic and facultatively anaerobic bacteria was isolated aerobically. These were composed of Vibrionaceae (1363 strains), *Pseudomonas* (84), *Micrococcus* (44), *Flavobacterium* (38), *Staphylococcus* (29), coryneforms (4), Enterobacteriaceae (1) and *Bacillus* (1). The Vibrionaceae were further divided into 20 groups on the basis of 11 characteristics, as shown in Table 1. Their occurrence (number of strains isolated) in each of the crustacean species examined is also shown in Table 1.

A total of 1024 strains of anaerobic bacteria were isolated anaerobically. These were obligate anaerobes (51 strains) and facultative anaerobes (973). The obligate anaerobes consisted of Gram-positive cocci (29), Gram-negative asporogenous rods (21) and a Gram-positive asporogenous rod (1).

Table 1. Grouping of the bacteria belonging to genus *Vibrio* isolated from coastal crustacea

Character	Group																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Sensitivity to O/129	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pigmentation	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Swarming	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Growth at 37°C	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—
Indole production	+	+	+	+	+	+	—	—	—	—	—	—	+	+	+	+	—	—	—	—
Colony on TCBS* ¹	Y	G	G	Y	G	—	Y	Y	G	B	—	—	Y	G	G	W	Y	G	—	—
Acid from:																				
Sucrose	+	—	—	+	—	—	+	+	—	—	—	—	+	—	—	—	+	—	+	—
Arabinose	—	+	—	—	—	—	+	—	—	—	—	—	—	+	—	—	—	—	—	—
Inositol	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
VP reaction	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	—	+	+	+	+	+	+	+	+	—
Crustacean species																				
Japanese spiny lobster	69* ²	0	0	75	200	2	0	7	12	3	0	0	55	0	42	0	3	14	7	0
<i>Atergatis</i> crab	0	0	0	2	9	0	0	0	2	0	0	0	42	0	70	0	3	14	9	0
<i>Schizophrys</i> crab	0	0	0	2	18	0	0	0	7	0	1	0	10	0	68	3	1	1	0	3
<i>Tiarinia</i> crab	0	0	0	1	15	0	0	0	7	0	1	0	28	2	50	0	1	11	0	1
<i>Pachygrapsus</i> crab	0	0	0	27	5	1	0	0	2	0	0	4	21	0	12	0	0	5	0	0
<i>Thalamita</i> crab	9	2	2	69	2	10	0	0	8	0	2	0	27	0	0	0	0	0	0	0
<i>Plagusia</i> crab	38	0	0	38	11	0	3	4	3	0	0	0	99	0	84	0	0	4	0	0
Total	116	2	2	214	260	13	3	11	41	3	4	4	282	2	326	3	8	49	16	4

*¹ Y, yellow; G, green; B, brown; W, white; —, no growth.*² Number of strains.

Table 2. Specific viable counts of bacteria in the gills and guts of Japanese spiny lobster

Component	Gills (n=5)	Gut (n=5)
<i>Vibrio</i> group 1	5.52±0.18 (100)* ¹	7.09±0.30 (40)
group 4	5.46±0.76 (100)	7.26±0.83 (100)
group 5	5.65±0.38 (100)	8.12±0.57 (100)
group 6	4.82±0.74 (40)	nd* ² (0)
group 8	5.79±0.64 (60)	5.60 (20)
group 9	4.13±1.60 (60)	7.86±0.91 (60)
group 10	5.00 (20)	nd (0)
group 13	5.49±0.85 (80)	6.56±0.83 (100)
group 15	4.81±0.86 (100)	6.41±1.80 (100)
group 17	3.60 (20)	5.30 (20)
group 18	6.00±0.35 (60)	7.07±0.68 (60)
group 19	4.45±0.21 (40)	nd (0)
<i>Pseudomonas</i>	6.19±0.33 (100)	6.60 (20)
<i>Flavobacterium</i>	4.62±1.35 (100)	nd (0)
<i>Staphylococcus</i>	nd (0)	7.20±0.24 (40)
<i>Micrococcus</i>	5.18±0.93 (60)	nd (0)
Coryneforms	4.80±0.71 (40)	6.60 (20)
Bacteroidaceae	4.30 (20)	nd (0)
Anaerobic	nd (0)	6.95±0.49 (40)
Gram-positive cocci		
TVC* ³	6.79±0.23	8.61±0.45

*¹ Mean±standard deviation of log maximum bacterial counts per gram (SVC) when present (frequency of occurrence among lobster specimens, %).

*² Not detected.

*³ Mean±standard deviation of log TVC per lobster specimen.

Microflora of Gills and Gut Contents of Coastal Crustacea

Viable counts of various groups of bacteria and TVCs in the gills and gut contents of the specimens of Japanese spiny lobster are given in Table 2. The lobsters were collected in October at which time water temperature was 20.8°C.

In the gills of the lobster, TVCs ranged from 10⁶ to 10⁷ cells g⁻¹, and 17 bacterial components were isolated. *Vibrio* were divided into 12 groups. *Vibrio* groups 1, 4, 5 and 15, *Pseudomonas* and *Flavobacterium* were isolated from all five lobsters at bacterial densities of 10² to 10⁶ cells g⁻¹. Anaerobes (Bacteroidaceae) were isolated from one of the 5 specimens.

In the guts of the lobsters described above, TVCs ranged from 10⁷ to 10⁹ cells g⁻¹, and 13 bacterial components were detected. Of 9 groups of *Vibrio* isolated, groups 4, 5, 13 and 15 were ubiquitous to all specimens with bacterial densities of 10³ to 10⁸ cells g⁻¹. Anaerobes (Gram-positive cocci) were isolated from two individuals at bacterial densities of 10⁶ to 10⁸ cells g⁻¹.

Gut microflora of 6 coastal crabs is summarized in Table 3. TVCs ranged from 10⁵ to 10⁸ cells g⁻¹.

From *Atergatis* crabs collected in March at

13.8°C, 13 bacterial components were isolated. Of the 5 *Vibrio* groups detected, only group 18 occurred in all samples. In the crab samples collected in April at 17.0°C, 10 bacterial components were detected. Of five groups of *Vibrio* isolated, groups 5, 13 and 15 occurred in all three individuals. The bacterial components isolated from 2 of the 3 specimens were *Vibrio* group 4, *Pseudomonas* and *Staphylococcus*. Anaerobes (Gram-positive cocci) were detected in only one crab collected in April.

From *Schizophrys* crabs collected in April at 17.0°C, 15 bacterial components were isolated, 10 of which were *Vibrio* groups. Only *Vibrio* group 15 was common to all five crab specimens. *Vibrio* group 13 and *Pseudomonas* each occurred in 4 of the 5 specimens. No anaerobes were detected in this type of crab.

In *Tiarinia* crabs collected in April at 17.0°C, 14 bacterial components containing 10 groups of *Vibrio* were detected. *Vibrio* groups 13 and 15 each occurred in all four crabs with bacterial densities of 10⁵ to 10⁷ cells g⁻¹. Anaerobes (Gram-positive cocci) were isolated from two crab samples.

From *Pachygrapsus* crabs collected in June at 21.7°C, 14 bacterial components were isolated,

Fig. 1. Growth temperature spectra of the isolates from the gut contents of *Plagusia* and *Atergatis* crabs. a, *Plagusia* crabs caught in March (13.8°C); b, in August (26.0°C); c, *Atergatis* in March (13.8°C); d, in April (17.0°C).

including 8 groups of *Vibrio*. *Vibrio* groups 4, 5, 13 and 18, *Flavobacterium*, *Micrococcus* and anaerobic asporogenous Gram-positive rods were detected in 60 to 80% of the specimens examined. In addition to the anaerobic rod, Gram-positive cocci were isolated from two crabs as anaerobic components.

In *Thalamita* crabs collected in August at 26.0°C, 13 bacterial components containing 9 groups of *Vibrio* were detected. Only *Vibrio* group 4 were common to all five crabs. No anaerobes were isolated from these animals.

From the *Plagusia* crabs collected in March at 13.8°C, 6 bacterial groups were isolated. *Vibrio* consisted of 3 groups, of which only group 13 was present in all five crabs. In the crabs collected in August at 26.0°C, 9 bacterial components containing 7 groups of *Vibrio* were detected. *Vibrio* groups 1, 4 and 5 were present in all three crabs. No anaerobes were isolated from *Plagusia* crabs in either March or August.

Fig. 1 shows the growth spectra of the aerobic bacteria from *Plagusia* and *Atergatis* crabs at temperatures ranging from 5 to 42°C. The isolates from *Plagusia* crabs collected in March (water temperature of 13.8°C) could not grow at 37°C but about 70% of the isolates grew at 5°C. However, almost all strains isolated from the crab in August (water temperature of 26.0°C) could grow at 37°C but not at 5°C. These results suggested that differences in taxonomic composition

of the gut microflora of this crab between March and August may be related to shifts in the ambient water temperature. A similar, though less marked, shift in temperature growth responses was detected for isolates from *Atergatis* crabs collected in March versus April, during which time a similar, though less marked, shift in ambient water temperature (from 13.8 to 17.0°C) also occurred.

Discussion

In the present study, three types of anaerobic bacteria, the Gram-positive coccus, Gram-positive asporogenous rod and Gram-negative asporogenous rod, were isolated from coastal crustacea. Although various types of anaerobes have been known to exist in the gastrointestinal tract of freshwater vertebrates, including fishes,^{11,12,15)} frogs^{14,21)} and soft-shelled turtles,¹³⁾ there is little information on the anaerobes associated with marine animals. Shewan²²⁾ isolated anaerobic sporeforming bacteria from haddock *Gadus aeglefinus* and the authors^{10,23)} reported the presence of clostridia and Bacteroidaceae in coastal bivalves and fishes. Additionally, Dyrset *et al.*²⁴⁾ isolated Bacteroidaceae from capelin *Mallotus villosus*. However, the isolation of anaerobic bacteria from marine crustacea has not been reported. This paper is the first demonstration that crustacea can have obligate anaerobes in their guts and gills. When detected, these

Table 3. Specific viable counts of different components of bacteria in the gut contents of six coastal crabs

Component	<i>Atergatis</i> (Mar.) (n=3)		<i>Atergatis</i> (Apr.) (n=3)		<i>Schizophrys</i> (n=5)		<i>Tiarinia</i> (n=4)	
<i>Vibrio</i> group 1	nd*	(0)* ²	nd	(0)	nd	(0)	nd	(0)
group 2	nd	(0)	nd	(0)	nd	(0)	nd	(0)
group 3	nd	(0)	nd	(0)	nd	(0)	nd	(0)
group 4	nd	(0)	6.37±0.59 (67)		5.54±0.24 (40)		6.60	(25)
group 5	nd	(0)	6.24±0.69 (100)		7.58 (20)		7.33	(25)
group 6	nd	(0)	nd	(0)	nd	(0)	nd	(0)
group 7	nd	(0)	nd	(0)	nd	(0)	nd	(0)
group 8	nd	(0)	nd	(0)	nd	(0)	nd	(0)
group 9	nd	(0)	6.25 (33)		6.60 (20)		5.30	(25)
group 11	nd	(0)	nd	(0)	6.26 (20)		5.30	(25)
group 12	nd	(0)	nd	(0)	nd	(0)	nd	(0)
group 13	6.60±1.84 (67)		7.20±0.42 (100)		6.04±0.62 (80)		6.50±0.83 (100)	
group 14	nd	(0)	nd	(0)	nd	(0)	5.60	(25)
group 15	8.02±0.56 (67)		7.15±0.60 (100)		7.05±1.01 (100)		6.96±0.60 (100)	
group 16	nd	(0)	nd	(0)	6.41 (20)		nd	(0)
group 17	8.25±0.23 (67)		nd	(0)	6.08 (20)		6.90	(25)
group 18	6.95±1.55 (100)		nd	(0)	6.60 (20)		6.71±0.37 (50)	
group 19	6.30 (33)		nd	(0)	nd	(0)	nd	(0)
group 20	nd	(0)	nd	(0)	5.78 (20)		5.30	(25)
<i>Pseudomonas</i>	5.95±0.92 (67)		6.60±0.30 (67)		6.77±0.40 (80)		6.10±0.20 (50)	
<i>Flavobacterium</i>	nd	(0)	nd	(0)	6.30 (20)		nd	(0)
Enterobacteriaceae	nd	(0)	nd	(0)	5.30 (20)		nd	(0)
<i>Staphylococcus</i>	7.01 (33)		5.69±0.09 (67)		nd	(0)	5.30	(25)
<i>Micrococcus</i>	nd	(0)	6.30 (33)		5.60±0.30 (40)		5.80±0.50 (50)	
Coryneforms	nd	(0)	nd	(0)	nd	(0)	nd	(0)
<i>Bacillus</i>	nd	(0)	4.34 (33)		7.30 (20)		nd	(0)
Anaerobic Gram-positive rods	nd	(0)	nd	(0)	nd	(0)	nd	(0)
Anaerobic Gram-positive cocci	nd	(0)	4.83 (33)		nd	(0)	5.56±0.19 (50)	
TVC* ³	7.91±1.25		7.77±0.04		7.64±0.75		7.54±0.32	

*1 Not detected. *2 Mean±standard deviation of log maximum bacterial number per gram (SVC) when present (frequency of

anaerobes were present at bacterial densities up to 10^7 cells g^{-1} . It is possible that their occurrence is restricted to certain species of crustacea, since anaerobes were never isolated from *Schizophrys*, *Thalamita* or *Plagusia* crabs, in the latter case regardless of water temperature. The low frequency of isolation of these bacteria may imply that they are transient forms in coastal crustacea. At present, these problems remain unresolved.

Nevertheless, the presence of these anaerobes is of much interest, because the information on the isolation of anaerobes (except for clostridia and Bacteroidaceae) from marine environments has been scarce. A detailed study concerning taxonomy and ecology of these organisms is now in progress.

The Japanese spiny lobster and *Plagusia* crab

guts appear to support significantly higher bacterial populations than the other crab guts or the lobster gills. Similarly high TVCs of gut contents were also observed in fishes.^{15, 23)} The difference of TVCs of crustacean guts could be due to diet, physiological condition and species of host animal, and time lapse of digestion. At present, however, it remains unclear which is the major factor.

It has been known that microflora in the gut of crustacea is mainly composed of *Vibrio*, *Pseudomonas* and coryneforms as described already. In the present study, *Vibrio* and *Pseudomonas* were isolated from seven different species of marine crustacea. The *Vibrio* strains were further divided into 20 groups on the basis of 11 additional characters. Sizemore et al.,⁹⁾ Sochard et al.,²⁾ and Atlas et al.³⁾ classified the *Vibrio* strains isolated from blue crab, copepoda or amphipoda,

Table 3. (Continued)

Component	<i>Pachygrapsus</i> (n=5)	<i>Thalamita</i> (n=5)	<i>Plagusia</i> (Mar.) (n=5)	<i>Plagusia</i> (Aug.) (n=3)
<i>Vibrio</i> group 1	nd (0)	5.92±0.23 (60)	nd (0)	8.33±0.41 (100)
group 2	nd (0)	5.60 (20)	nd (0)	nd (0)
group 3	nd (0)	6.30 (20)	nd (0)	nd (0)
group 4	6.32±1.21 (80)	6.91±0.62 (100)	nd (0)	7.86±0.32 (100)
group 5	5.73±0.42 (60)	6.03±1.03 (60)	nd (0)	6.97±0.47 (100)
group 6	nd (0)	6.05±0.55 (60)	nd (0)	nd (0)
group 7	nd (0)	nd (0)	nd (0)	7.58 (33)
group 8	nd (0)	nd (0)	nd (0)	7.30 (33)
group 9	5.80±0.50 (40)	7.60 (20)	nd (0)	7.45 (33)
group 11	nd (0)	5.95±0.58 (40)	nd (0)	nd (0)
group 12	5.60 (20)	nd (0)	nd (0)	nd (0)
group 13	6.19±0.25 (60)	6.58±0.60 (80)	7.70±1.48 (100)	7.25±0.05 (67)
group 14	nd (0)	nd (0)	nd (0)	nd (0)
group 15	7.34 (20)	nd (0)	8.22±0.67 (80)	nd (0)
group 16	nd (0)	nd (0)	nd (0)	nd (0)
group 17	nd (0)	nd (0)	nd (0)	nd (0)
group 18	6.20±0.70 (60)	nd (0)	6.54±0.34 (40)	nd (0)
group 19	5.30 (20)	nd (0)	nd (0)	nd (0)
group 20	nd (0)	nd (0)	nd (0)	nd (0)
<i>Pseudomonas</i>	6.70±0.40 (40)	5.63±0.41 (80)	6.87±0.51 (60)	nd (0)
<i>Flavobacterium</i>	6.62±0.69 (80)	5.95±0.35 (40)	6.97±0.26 (40)	nd (0)
Enterobacteriaceae	nd (0)	nd (0)	nd (0)	nd (0)
<i>Staphylococcus</i>	6.99±0.61 (40)	6.19±0.11 (40)	nd (0)	7.60 (33)
<i>Micrococcus</i>	5.79±0.50 (60)	6.56±0.78 (40)	nd (0)	8.38 (33)
Coryneforms	nd (0)	nd (0)	5.30 (20)	nd (0)
<i>Bacillus</i>	nd (0)	nd (0)	nd (0)	nd (0)
Anaerobic Gram-positive rods	5.80±1.07 (80)	nd (0)	nd (0)	nd (0)
Anaerobic Gram-positive cocci	6.32±0.95 (40)	nd (0)	nd (0)	nd (0)
TVC* ³	7.65±0.43	7.49±0.47	8.45±0.64	8.66±0.25

occurrence among crab specimens, %). *³ Mean±standard deviation of log TVC per crab specimen.

into various phena using numerical taxonomy. At present, it remains unresolved whether each group of *Vibrio* can be further classified into different species of *Vibrio*. In any case, it is clear from this study that the microflora of crustacea consists of many different bacterial components. One reason for the detection of this diversity may be the use of six different agar media for aerobic cultivation, because the effectiveness of isolating the bacteria varied with the medium used. Similar results were also obtained in a study of the bacterial populations in water, sediments and fish guts of freshwater environments.²⁵⁾ These results suggest that the combination of non-selective and selective media allows a more complete investigation of the gut microflora of aquatic animals, as has already been shown for the homeothermal animals studied by Mitsuoka *et al.*²⁶⁾

Tables 1 to 3 show that *Vibrio* groups 4, 5, 9, 13,

15 and 18, *Pseudomonas*, *Staphylococcus*, and *Micrococcus* are widely distributed in the crustacea. These bacterial components may be specific to coastal crustacea, but other bacteria were found in selected species, individual specimens, or specimen tissues.

A total of 17 components of bacteria was found in the gills of the Japanese spiny lobster while the microflora of their guts comprised 13 bacterial components, all of which were present in the gills. Liston²⁷⁾ reported that the diverse nature of the bacterial populations of skin and gills of fish is probably due to the ease of access of these sites of growth from the surrounding water and to the generally favorable conditions of growth provided by the slime secreted continuously from fish. Perhaps the diversity of gill microflora in crustacea is also affected by bacterial diversity in the surrounding water. With regards to the bacterial

gut populations, some components may be inhibited by the characteristically low pH, bile acids or anaerobic conditions in the gut, resulting in the dominance of certain *Vibrio* groups, as known in marine fish.^{28,29)}

It was found from Table 3 that the gut microflora of *Atergatis* and *Plagusia* crabs varied significantly with sampling time. As shown in Fig. 1, the presence of isolates capable of growth at 5 and/or 37°C differed markedly between the *Plagusia* crabs collected in March at 13.8°C and in August at 26.0°C. This result is perhaps a reflection of shift in surrounding water temperature. In the case of *Atergatis* crabs, the growth temperature spectra of their bacteria in March is similar to that of *Plagusia* bacteria in the same month, whereas the *Atergatis* bacteria in April at 17.0°C seem to be transient, or just beginning a shift from spring to summer composition. These results suggest that the gut microflora of coastal crustacea is easily influenced by the surrounding water temperature. In any case, the gut microflora of coastal crustacea might not be as stable as that observed in fishes.¹⁾

It has been recently recognized that intestinal microflora of homeothermal animals play important roles in nutrition, carcinogenesis and self-defense of the host.³⁰⁻³²⁾ Although it is believed that similar relationships occur in aquatic animals, there is a lack of evidence and only a few studies on this relationship have been reported.³³⁻³⁶⁾ Therefore, further studies of the gut microflora of crustacea concerning this problem should be performed.

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