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Microflora in the Digestive Tract of Marine Fish-I

General Characterization of the Isolates from Yellow Tail

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Abstract

The bacterial flora in the digestive tract, especially in the intestine, of yellow tail (*Seriola quinqueradiata*) which had been cultured in a commercial fish crawl was occupied by *Vibrio* species at the high frequency as compared with that of sea water samples. Intestinal *Vibrio* species isolated in this experiment were the slight halophilic type bacteria which were able to grow well at 37 C and resistant to low pH (pH 4.5) and bile salts. Most of them were able to hydrolyze chitin but unable to hydrolyze casein, gelatin and starch.

It has been established that the indigenous microflora of the digestive tract of fish is made up of microorganisms which are entirely different from those of the body surface and gills and that the microflora of fish intestine also differs from gastrointestinal microflora of mammals. LISTON[1] reported that while the gut group *Vibrios* predominated in the digestive tract, *Pseudomonas* and *Achromobacter* were commonly found in the body surface and gills of fish from North Sea. A number of other workers[2)~5)] supported the occurrence of *Vibrios* specific in the digestive tract of marine fishes. YOSHIMIZU et al.[6)7)] showed that the intestinal microflora of salmonids are mainly composed of the genus *Aeromonus* and family *Enterobacteriacea*, if they are living in fresh water, on the other hand the flora are mainly composed of the genus *Vibrio* when living in sea water.

Recently various problems in nutrition and disease of fish grow up as the commercial culture of fish has developped. Much attention has been given to the significance of intestinal microflora for nutrition and immunological systems of fish as well as mammals. From these aspects it is necessary to obtain the more information on the characteristics and activities in physiology and ecology of intestinal microflora of fish. This paper describes the characterization of isolates from the digestive tract of yellow-tail cultured in a commercial fish crawl.

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Materials and Methods

Fish studied. Yellow-tail (*Seriola quinqueradiata*) sampled in this investigation had been cultured in a commercial fish crawl at Kinko Bay, Kagoshima Prefecture. The fishes were fed with minced meat of mackerel or sardine. The fish samples, which were 2 years of age and 1.3–2.0 Kg in body weight, were brought to the laboratory immediately after being captured by fish net.

Bacterial counts. The ventral surface of the fish samples was thoroughly washed by the sanitary cotton impregnated with 70% alcohol. After open the ventral surface by a dissecting knife, the digestive tract was taken out and separated into stomach, pyloric caeca and intestine. Each part of the digestive tract was transferred to the mortar and homogenized aseptically. The homogenized samples of the digestive tract and sea water samples which obtained from outer (300 m off) and inner region of the fish crawl, were diluted with half strength and full strength artificial sea water (ASW) respectively. Viable bacteria present were enumerated on agar plate by the smear plate method.

Media used and cultural conditions. M-BII medium, which was developped after modified medium B described by SIMIDU[8] for the isolation of heterotrophs from marine fish, contained 1.0% polypeptone (Daigo Eiyo), 0.3% yeast extract (Daigo Eiyo) and half strength ASW (Herbst's formmula). The final pH was adjusted to 7.5 with NaOH. For the bacteria isolated from sea water ZoBell 2216E medium was employed as a basal medium, in which ASW was used instead of natural sea water. Enumaration of anaerobic bacteria was made with the following method. Each portion (1 ml) of suitable dilutions was added to test tubes with 20 ml thioglycolate medium (Eiken) which had been held at 45C. The test tubes were capped with tightly fitting rubber stoppers and mixed thoroughly by an electric vibrator. Immediately, inoculated media were transferred into a desiccator with N_2 gas and alkaline pyrogallol. BGLB (Nissan) agar medium was used for examination of sensitivity to gall powder. Inoculated media were incubated at 25 C for one week aerobically or anaerobically.

Bacteriologicl examination. About 50 bacterial strains were picked up from a plate of suitable dilution which contained 50–300 colonies. The isolates were then purified and maintained on M-BII medium and ZoBell medium for gastrointestinal bacteria and marine bacteria respectively. The characterizations of isolates were ascertained according to the standard methods described by HARRIGAN et al.[9]. Identification and classification of isolates were based on the scheme of SHEWAN[10] and its modification proposed by SIMIDU[11]. Behavior of isolates for salts requirement was examined according to the method proposed by HIDAKA and SAKAI[12]. The abilities to hydrolyze macromolecules were confirmed by determining the clear zone which was observed on agar plate during incubation. Test plate for hydrolysis of macromolecules consisted of a basal medium and one of various substrates, such as casein (0.5%), gelatin (1.0%), alginate

(0.75%), tributyrin (1.0%), starch (0.5%), chitin (0.5%) and cellulose (0.5% w/v).

Effect of pH and bile salt on bacterial growth. Effect of pH and bile salt on bacterial growth was examined both on agar plate and in liquid medium. In the liquid medium, the bacterial growth was estimated spectrophotometrically by the optical density at 540 nm after incubated at 25 C for 5 days. The turbidity of culture at pH 4.5 (initial) or with 0.5% taurocholate (Nakarai) was compared with that of basal medium (pH 7.5 and without taurocholate).

Results

Viable counts in the digestive tract. The viable counts in the digestive tract of yellow-tail and environmental sea water are shown in Table 1. The digestive tract contained viable aerobes from 6.5×10^4 to 5.9×10^6 per gram wet weight of samples which consisted of the fish digestive tract and its contents. The highest count was obtained in intestine and the lowest in pyloric caeca. In the case of fish intestine, almost same number of bacteria were able to grow aerobically and anaerobically and the greater part of bacteria was resistant to low pH (pH 4.5) and BGLB medium. On the other hand, sea water samples contained viable cells from 2.7×10^2 to 1.0×10^3 which were able to scarcely grow anaerobically, at pH 4.5, or on BGLB medium.

Table 1. Viable Counts in the Digestive Tract and Sea Water

Source	Viable counts c. f. u./g, ml				
	Expt. 1 (Oct.)	Expt. 2 (Feb.)			
	pH 7.5 Aerobic	pH 7.5 Aerobic	pH 7.5 Anaerobic	pH 4.5 Aerobic	BGLB Aerobic
Stomach	2.6×10^5	2.3×10^5	—	1.1×10^4	1.7×10^5
Pyloric caeca	6.5×10^4	2.0×10^4	—	—	—
Intestine	5.9×10^6	6.6×10^5	7.1×10^5	3.1×10^5	5.6×10^5
Diet	—	1.6×10^5	1.8×10^4	3.0×10^3	1.1×10^5
Sea water A	1.0×10^3	4.8×10^2	—	0	0
Sea water B	8.2×10^2	2.7×10^2	$>10^1$	$>10^1$	$>10^1$

Generic composition of the microflora. As shown in Table 2 the percentage of *Vibrio* species in the digestive tract was very high. Especially in intestine 90% of the isolates were identified as species of *Vibrio* and the genus *Pseudomonas*, *Acinetobacter* and *Flavobacterium* were scarcely isolated although they were commonly found in sea water. Sea water sample B which was obtained at the inner region of a fish crawl, contained higher ratio of *Vibrio* compared with sea water sample A in which a wide range of species was distributed.

Effect of low pH and bile salts. The ability of isolates to grow on the medium

Table 2. Generic Composition of Bacterial Flora in the Digestive Tract and Sea Water

Genera	Percentage of isolates				
	Digestive tract			Sea water	
	Stomach	Pyloric caeca	Intestine	A	B
Vibrio	69	80	91	18	79
Aeromonas	4	4	5	0	0
Pseudomonas	6	4	0	20	19
Acinetobacter	0	0	0	12	0
Flavobacterium	8	8	4	14	0
Achromobacter	0	2	0	0	0
Coccus	8	0	0	28	0
Not identified	5	2	0	8	2
Total strains	49	50	50	50	52

Table 3. Effect of Gall Powder and Low pH on Bacterial Growth

Source	Percentage of resistants			Total strains
	Gall powder 0.2%	Gall powder 2.0%	Low pH (pH 4.5)	
Stomach	82	80	82	45
Pyloric caeca	94	90	92	48
Intestine	100	98	96	50
Sea water A	42	10	16	50
B	69	6	35	52

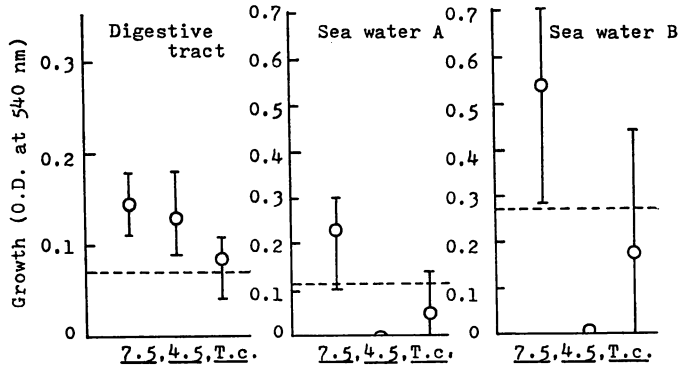

Fig. 1. Effect of Low pH and Taurocholate on Bacterial Growth. Dotted lines indicate the half level of optical density obtained at pH 7.5.

containing bile salts (2% bovine gall powder or 0.5% taurocholate) or at low pH (pH 4.5) was examined. The results in Table 3 and Fig. 1 indicate that the isolates from the digestive tract were relatively resistant to low pH and

bile salts as compared with those from sea water.

Salts requirement for growth. As shown in Table 4 and Fig. 2, the isolates from the digestive tract had an optimal NaCl concentration for growth between 2 and 3% and somewhat lower than that of marine bacteria. A large number of isolates from the digestive tract belonged to the slight halophilic type (H-L) which can grow both in 0.5% and 3.0% NaCl media. On the other hand the isolates from sea water belonged to either marine (M) or halophilic (H-H) type.

Table 4. Bacterial Typing according to the Mineral Requirement

Source	Bacterial type				Total strains
	M	H-H	H-L	T	
Stomach	6	13	72	9	47
Pyloric caeca	2	6	90	2	50
Intestine	0	4	96	0	50
Sea water A	67	31	0	2	49
B	23	77	0	0	52

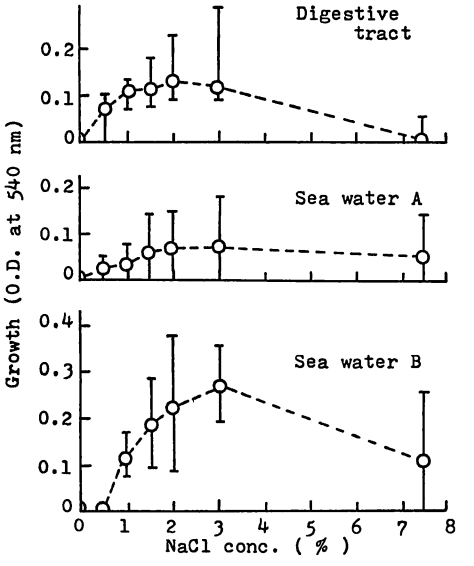

Fig. 2. Effect of NaCl Concentration on Growth.

Hydrolytic activities of macromolecules. Table 5 shows the results of hydrolytic activities of various macromolecules. Intestinal isolates had the activity to hydrolyze chitin but not to hydrolyze casein, gelatin and starch. On the other hand the isolates possessing the hydrolytic activity of casein and gelatin from sea water were more abundant than the chitin-decomposing ones.

Table 5. Hydrolytic Activities of Isolates on Various High Molecular Compounds

Compound	Percentage of positive strains				
	Digestive tract			Sea water	
	St.	P. c.	In.	A	B
Casein	4	0	0	64	79
Gelatin	8	2	0	60	79
Alginate	0	0	0	2	56
Tributyrin	92	98	100	86	100
Starch	2	0	0	6	40
Chitin	58	96	100	38	62
Cellulose	0	0	0	0	0
Total strains	50	48	50	50	52

Discussion

Many investigators reported that the genus *Vibrio* predominate in the intestine of various marine fishes. For example LISTON[1] indicated the occurrence of the gut group *Vibrio* and SERA et al.[3] suggested that indigenous bacterial flora in the digestive tract of various marine fishes was occupied by a specific *Vibrio* group which was uniquely resistant to bile and low pH.

Table 6. Main Characteristics of Dominant Strains from Intestine

Cell from	Rod	Arginine dihydrolase	+
Gram stain	−	Indole production	−
Motility	+	Nitrate reduction	+
Flagellation	M	V. P. test	+
Hugh and Leifson test	F	M. R. test	+
Cytochrome oxidase	+	Hydrolysis of casein	−
Catalase	+	gelatin	−(+)
Growth at 37 C	+	alginate	−
Growth at pH 4.5	+	tributyrin	+
M, H, T, typing	H-L	starch	−
Sensitivity to 0/129	+	chitin	+
H_2S production (SIM medium)	−	cellulose	−

The present results on yellow tail also indicate that the genus *Vibrio* is isolated at the high percentage (90%) in the intestine of yellow tail. Common characteristics of *Vibrio* species isolated from the intestine are summarized in Table 6. Intestinal *Vibrio* species can grow at 37 C and at low pH (pH 4.5). They are slight halophilic type (H-L) and hydrolyze chitin only while do not casein, gelatin and starch. These characters agree with those of a specific *Vibrio* group from sea bream described by SERA et al.[3]. In the aspects of physiology

and nutrition of host fish, it is interesting that the intestinal *Vibrio* species are unable to hydrolyze important macromolecules such as casein, gelatin and starch except chitin. *Vibrio* species with the characters presented above were detected at relatively high percentage from in diet (mackerel minced meat) and in inner crawl. Intestinal *Vibrios* are suggested to be derived through food chain from diet or environmental sea water and colonize in intestine after selected by various mechanismas. As these selective mechanisms, low pH in stomach or bile salts and anaerobic condition in intestine should be taken into consideration. However it is necessary to examine the selective mechanisms for intestinal microflora in detail.

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