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Taxonomical and Serological Studies on the Causative Bacteria of the Disease of Sea Urchin *Strongylocentrotus intermedius* Occurring at Low Water Temperatures

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Identification of the causative bacteria *Vibrio* spp., which were isolated from diseased sea urchin *Strongylocentrotus intermedius* and their rearing cages at low seawater temperatures at Date, Shiriuchi and Shikabe Fisheries Breeding Centers in Hokkaido was undertaken based on their biochemical properties, DNA-DNA homology and serological analysis. All fifteen strains, five from each Breeding Center were almost identical in biochemical properties except indole production. However, the strains isolated at Date differed from the strains at Shiriuchi and Shikabe in DNA-DNA homology and analysis of thermostable and thermolabile antigenic compositions. These results indicated that the strains from Shiriuchi and Shikabe belonged to the same species but they were different from those of Date. Moreover, the causative *Vibrio* spp. differed in biochemical, genotypical and serological properties from any strains of *Vibrio* spp. isolated from the intestine of healthy sea urchin and any of type strains of the genus *Vibrio*.

Key words: sea urchin, bacterial disease, identification, *Vibrio* spp., low water temperatures

There are so far several reports about mass mortalities of cultured sea urchins in Japan.[1,2] Kanai*2 reported that mass mortalities occurred in cultured sea urchins *Pseudocentrotus depressus* and *Hemicentrotus pulcherrinus* in the winter season (seawater temperature was about 14°C) in Kyushu in 1993 and he isolated a gliding bacterium as the aetiological agent. In 1995, mass mortalities have occurred in cultured sea urchin *Strongylocentrotus intermedius* at low water temperature (seawater temperature ranged 11-13°C) at Fisheries Breeding Centers in southern parts of Hokkaido. It was the first outbreak in cultured sea urchin in Hokkaido at low water temperatures. We isolated the causative bacteria belonging to the genus *Vibrio* from coelomic fluid and body surface of diseased sea urchins as described in the previous paper.[3] In this work, we tried to determine the taxonomic status of *Vibrio* strains by comparing them with the reference strains of the genus *Vibrio*. Moreover, thermostable and thermolabile antigenic compositions of the isolates were analysed.

Table 1. Sources of bacterial strains used in this study

Fisheries Breeding Center	Strain No.	Source
Date	Da-1	Coelomic fluid of diseased sea urchin
	-2	〃
	-3	Body surface of diseased sea urchin
	-4	〃
	-5	Rearing cage of diseased sea urchin
Shiriuchi	Sr-1	Coelomic fluid of diseased sea urchin
	-2	〃
	-3	〃
	-4	Body surface of diseased sea urchin
	-5	Coelomic fluid of diseased sea urchin
Shikabe	Sk-1	〃
	-2	〃
	-3	〃
	-4	〃
	-5	〃

Materials and Methods

Bacterial Strains

Fifteen strains isolated from diseased sea urchins which were tentatively identified to the genus *Vibrio*,[3] 17 reference strains of the genus *Vibrio* and 15 strains of *Vibrio* spp. isolated from the intestine of healthy sea urchins were used in this study as shown in Tables 1 and 2. All strains were maintained on seawater agar medium (SA: polypepton 1 g, yeast extract 1 g, proteose peptone 1 g, meat extract 1 g, 75% Herbst's artificial seawater[4] 1000 m*l*, pH 7.8). SA medium was also used as the basal medium for determination of biochemical properties.

Biochemical and Physiological Characteristics of Isolates

The biochemical and physiological characteristics of the strains including the reference strains were investigated according to the methods described by Sakazaki:[5] pigmentation, oxidase, catalase, Na^+ requirement, arginine dihydroxylation, lysine and ornithine decarboxylation, VP

*2 K. Kanai: "Togenukesho" of sea urchins. Proceedings of the Symposium on "Diseases in fish and shellfish culture in Kyushu and Okinawa". Japanese Society of Fish Pathology, 1993, p. 7.

Table 2. Reference strains used in this study

Strain	Strain No./Source
V. anguillarum biovar I	NCMB 6
V. anguillarum biovar II	ATCC 33509
V. proteolyticus	NCMB 1326
V. harveyi	NCMB 1280
V. campbellii	ATCC 25920
V. alginolyticus	V-447
V. parahaemolyticus	H-O-5
V. fischeri	ATCC 7744
V. splendidus	HUPF 9117 (=ATCC 33125)
V. pelagius	ATCC 25916
V. damsela	ATCC 33539
V. costicolus	NCMB 701
V. diazotorophicus	ATCC 33466
V. gazogenes	ATCC 29988
V. hollisae	JCM 01283
V. natriegens	CCM 2527
V. nereis	ATCC 25917
Vibrio spp.*	Ua1, Ua3, 15; Ud10, Ud13, Ud21 Uf1, Uf2, Uf3; Ug3, Ug6, Ug8 Un12, Un15, Un21
Photobacterium leiognathi	NCMB 391
Escherichia coli	Es1

* Isolated from intestine of healthy sea urchins (Sawabe *et al.*, 1995).
NCMB: National Collection of Marine Bacteria.
ATCC: American Type Culture Collection.
HUPF: Hiroshima University Fish Pathology Laboratory Culture Collection.
JCM: Japan Collection of Microorganisms.
CCM: Czechoslovak Collection of Microorganisms.

and MR tests, amylase, caseinase, lipase, gelatinase, DNase, hydrogen sulfide, indole production and acid from carbohydrates except alginase[6] and chitinase.[7]

DNA Base Composition and DNA-DNA Hybridization

DNAs from the strains were extracted and purified according to the method of Marmur[8] with some modifications. The G+C contents of DNAs were determined by the high performance liquid chromatography of their nuclease P1 hydrolysate, according to the method of Kumagai *et al.*[9] DNA-DNA hybridization was performed according to the method described by Ezaki *et al.*[10] with minor modifications. DNAs of the representative strains Da-2 (Date), Sr-3 (Shiriuchi) and Sk-1 (Shikabe) of the three Breeding Centers were labeled with photobiotin.

Serological Analysis

Rabbit antisera of three representative strains, Da-2 (Date), Sr-3 (shiriuchi) and Sk-1 (Shikabe) were prepared as described previously.[2] The cross-agglutination reactions were carried out to analyse thermostable and thermolabile antigenic compositions among three representative strains as previously described method.[3] Absorbed sera were prepared by adding saline washed heavy suspensions of heat-killed cells (O-antigen; cells were heated at 121°C for 20 min in an autoclave) or formalin-killed cells (F-antigen) to diluted (1:100) antisera. The mixture was incubated at 37°C for 2 h and then stored overnight at 4°C. The bacterial cells were removed by centrifugation and supernatant fluids (absorbed sera) were used to determine the thermostable and thermolabile antigenic compositions.

Results and Discussion

The causative bacteria isolated from diseased sea urchins of three Breeding Centers were Gram-negative, oxidase positive, facultative anaerobic, slightly curved short rod organisms with a single polar flagellum as described previously.[3] They fermented glucose without gas production. The G+C contents of all the 15 strains were ranging from 43.3 to 46.4 mol%. On the basis of these characteristics, we confirmed that the strains belong to the genus *Vibrio*. The biochemical and physiological characteristics of 15 isolated strains and the reference strains are shown in Table 3. Biochemical properties of the strains of Shiriuchi were completely identical with those of Shikabe. However, they differed from those of Date only by indole production test. There were no strains of each Breeding Center which were identified to the reference strains. However, *V. campbellii*, *V. proteolyticus*, *V. harveyi* and *Vibrio* spp. isolated from the intestine of sea urchin were relatively closer to the strains, even though they differed in 4 or 5 properties from our strains (Table 3).

All the strains of Date were genotypically closely related to the strain Da-2 with a homology values ranging from 80.6 to 100% (Table 4). But the strain Da-2 showed relatively low relatedness ranging from 44.1 to 56.4% with strains of Shiriuchi and Shikabe. On the other hand, the strains of Shiriuchi and Shikabe were also genotypically closely related to each other showing significant homology values from 78 to 100% (Table 4) but low relatedness (33.1 to 46.9%) with those of Date. These results indicated that the strains of Shiriuchi and Shikabe are genotypically identical, but they differed from those of Date in genospecies level.

Three representative strains (Da-2, Sr-3 and Sk-1) were different from those of 17 reference strains because of their relatively low homology values ranging from 0.9 to 25.1%. Three representative strains gave comparatively high homology values with those of intestinal *Vibrio* spp. of sea urchin ranging from 18.2 to 64.1% (Table 5).

The alginolytic strains[11] from the intestine of sea urchin were suspected as the causative organisms at early stage of the study, because our strains isolated from coelomic fluid of diseased sea urchin showed a high alginolytic activity too. But the results of DNA-DNA homology presented herein clarified that diseased agents were different from that of intestinal *Vibrio* spp.

The slide-agglutination reactions among anti-Da-2, -Sr-3 and -Sk-1 sera and formalin-killed cells of each strain isolated from three Breeding Centers indicated that the strains of Date were different from those of Shiriuchi and Shikabe and the strains of Shiriuchi were identical to Shikabe in serology also (Table 6). None of the reference strains (formalin-killed cells) reacted with any antisera by slide agglutination reactions (data not shown). This suggests that the causative organisms are different from the reference strains in serology also.

To determine thermostable and thermolabile antigenic compositions of the isolated strains the cross-agglutination reaction was carried out between antisera absorbed with homologous and heterologous antigens of the representative strains Da-2, Sr-3 and Sk-1, and their O or

Table 3. Some distinguishing characteristics between the strains from diseased sea urchin and reference strains

Characteristics	Date (5 strains)	Shiriuchi (5 strains)	Shikabe (5 strains)	*V. anguillarum* biovar I	*V. anguillarum* biovar II	*V. alginolyticus*	*V. campbellii*	*V. costicolus*	*V. damsela*	*V. diazotorophicus*	*V. fischeri*	*V. gazogenes*	*V. harveyi*	*V. hollisae*	*V. natrigens*	*V. nereis*	*V. parahaemolyticus*	*V. pelagius*	*V. proteolyticus*	*V. splendidus*	*E. coli*	*P. leiognathi*	Ua, Ud, Uf, Ug, Un (15 strains)
Pigment	−	−	−	−	−	−	−	−	−	−	−	R	−	−	−	−	−	−	−	−	−	−	−
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+(12)
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	+	−	+	+	+
Growth in 1% NaCl	−	−	−	+	−	+	−	−	+	−	−	−	−	−	−	−	−	+	+	+	+	−	+
Growth at 4°C	+	+	+	+	−	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	+
10°C	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	−	+
30°C	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37°C	−	−	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Arginine dihydrolase	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	+(8)
Lysine decarboxylase	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−
Ornithine decarboxylase	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
Voges-Proskauer test	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−
Methyl red test	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+
Alginase	+	+	+	+	−	+	+	−	−	−	+	−	+	−	−	−	+	+	+	−	−	+	+
Amylase	+	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	+	+	+	+	−	+	+
Caseinase	+	+	+	+	+	+	−	−	−	+	−	+	−	−	+	−	+	−	+	−	−	−	+(12)
Chitinase	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Lipase	+	+	+	+	+	−	+	−	+	−	+	+	+	+	+	−	+	+	+	+	−	+	+
Gelatinase	+	+	+	+	+	+	+	−	−	−	+	+	+	−	+	−	+	+	+	+	+	+	+
DNase	+	+	+	+	−	+	+	−	−	−	+	+	+	+	+	+	+	−	+	+	−	+	+
H_2S production	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−(8)
Indole production	+	−	−	+	−	+	+	−	−	+	−	+	+	+	−	+	+	−	+	+	+	+	+
Acid from L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−(14)
D-Cellobiose	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+	−	+	+	+	+	−	+	+
Dextrin	+	+	+	+	−	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+
Dulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
myo-Inositol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−
Lactose	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	+	+	−	+(12)
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	−	+	+	+
D-Mannose	+	+	+	−	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
Raffinose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−(8)
D-Sorbitol	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−(13)
Sucrose	−	−	−	+	+	+	−	+	−	+	−	+	+	−	+	+	+	+	−	+	+	+	+(12)
Trehalose	+	+	+	+	−	+	+	+	−	+	−	+	+	−	+	+	+	+	+	+	+	+	+
D-Xylose	−	−	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	−	−	+	+	−

( ): No. of strain.
R: red.

F-antigens. The results of the cross-agglutination reactions among anti-Da-2, -Sr-3 and -Sk-1 sera absorbed with heterologous O-antigens, and their O-antigens are shown in Table 7. The anti-Da-2 sera absorbed with O-antigens of the strains Sr-3 and Sk-1 reacted positively only with O-antigen of the strain Da-2. The anti-Sr-3 and -Sk-1 sera absorbed with O-antigen of the strain Da-2 reacted positively with both O-antigens of the strains Sr-3 and Sk-1. On the other hand, anti-Sr-3 and -Sk-1 sera absorbed with O-antigens of the strains Sr-3 and Sk-1 reacted negatively with both O-antigens of the strains Sr-3 and Sk-1 and vice versa. These results suggested that thermostable antigenic composition of the strain Da-2 is different from those of the strains Sr-3 and Sk-1 and that of the strains Sr-3 and Sk-1 were identical to each other. The results of the cross-agglutination reactions between anti-Da-2, -Sr-3 and -Sk-1 sera absorbed with homologous and heterologous O-antigens and their F-antigens are shown in Table 8. The anti-Da-2 sera absorbed with homo- and heterologous O-antigens reacted positively only with F-antigen of the strain Da-2. The anti-Sr-3 and -Sk-1 sera absorbed with homologous and heterologous O-antigens reacted all positively with both F-antigens of the strains Sr-3 and Sk-1 and vice versa. These results suggested that the strains Da-2, Sr-3 and Sk-1 have thermolabile antigens and that the strains Sr-3 and Sk-1 have common thermolabile antigens which are not in the strain Da-2. The results of the cross-agglutination reactions between anti-Da-2, -Sr-3 and -Sk-1 sera

Table 4. Guanine plus cytosine content (mol%) of DNA from the strains and DNA-DNA homology (%) against the strains Da-2, Sr-3 and Sk-1

Strain	GC mol%	DNA homology (%) with		
		Da-2	Sr-3	Sk-1
Da-1	44.3	86.3	35.1	35.1
-2	44.0	100.0	36.5	43.9
-3	44.1	91.3	37.4	46.9
-4	43.8	80.6	33.1	33.8
-5	44.0	87.1	35.5	41.3
Sr-1	46.3	44.6	86.6	99.2
-2	46.4	44.9	90.0	102.2
-3	43.5	56.4	100.0	92.8
-4	46.4	43.9	78.0	98.7
-5	43.6	52.8	78.7	110.2
Sk-1	44.3	51.0	78.6	100.0
-2	45.1	49.7	95.1	100.0
-3	43.3	46.2	84.4	103.5
-4	46.1	44.1	82.6	98.2
-5	46.1	49.4	80.9	94.7

Table 5. DNA-DNA homology (%) of the reference strains against the strains Da-2, Sr-3 and Sk-1

Strain	DNA homology (%) with		
	Da-2	Sr-3	Sk-1
V. anguillarum biovar I	8.6	10.4	6.1
V. anguillarum biovar II	8.1	8.7	6.6
V. proteolyticus	11.3	9.2	6.7
V. harveyi	14.8	12.9	13.2
V. campbellii	18.8	12.2	10.7
V. alginolyticus	16.2	11.7	5.9
V. parahaemolyticus	15.3	11.8	6.1
V. fischeri	11.4	8.0	5.4
V. splendidus	13.2	9.9	6.8
V. pelagius	25.1	17.8	22.7
V. damsela	9.8	7.3	8.4
V. costicolus	6.6	5.2	1.2
V. diazotorophicus	11.7	7.4	9.5
V. gazogenes	5.9	6.0	6.6
V. hollisae	3.0	9.1	0.9
V. natriegens	10.1	10.5	18.3
V. nereis	13.5	9.6	19.3
P. leiognathi	6.6	4.8	2.5
E. coli	2.5	2.4	0.8
Vibrio spp. Ua1	18.2	43.7	48.9
Vibrio spp. Ug3	46.4	57.4	50.9
Vibrio spp. Un12	61.0	51.0	64.1

absorbed homologous and heterologous F-antigens, and their F-antigens are shown in Table 9. The anti-Da-2 sera absorbed with F-antigens of the strains Sr-3 and Sk-1 reacted positively only with F-antigen of the strain Da-2. The anti-Sr-3 and -Sk-1 sera absorbed with F-antigen of the strain Da-2 reacted positively only with F-antigens of the strains Sr-3 and Sk-1 and vice versa. Here also the thermolabile antigenic compositions of strains Sr-3 and Sk-1 are identical, but different from that of the strain Da-2.

We therefore concluded that the strains Sr-3 and Sk-1 are different both genetically and serologically from the strain Da-2. Based on the comparative studies with reference *Vibrio* species, the strains isolated from diseased sea urchins seemed to be new species in the genus *Vibrio*.

Table 6. Slide agglutination reactions among anti-Da-2, Sr-3 and Sk-1 sera and formalin-killed cells of the strains isolated from diseased sea urchins at three Breeding Centers

Antigen (F)	Reaction to antiserum for		
	Da-2	Sr-3	Sk-1
Da-1	+	−	−
-2	+	−	−
-3	+	−	−
-4	+	−	−
-5	+	−	−
Sr-1	−	+	+
-2	−	+	+
-3	−	+	+
-4	−	+	+
-5	−	+	+
Sk-1	−	+	+
-2	−	+	+
-3	−	+	+
-4	−	+	+
-5	−	+	+

(F): formalin-killed cells.

Table 7. Cross-agglutination reactions of anti-Da-2, Sr-3 and Sk-1 sera after absorption with cells of heterologous O-antigen

Antiserum	Absorbing antigen(O)	Antigen		
		Da-2(O)	Sr-3(O)	Sk-1(O)
Da-2	Sr-3	+	−	−
	Sk-1	+	−	−
Sr-3	Da-2	−	+	+
	Sk-1	−	−	−
Sk-1	Da-2	−	+	+
	Sr-3	−	−	−

(O): heat-killed cells.

Table 8. Cross-agglutination reactions of anti-Da-2, Sr-3 and Sk-1 sera after absorption with cells of homologous and heterologous O-antigens

Antiserum	Absorbing antigen(O)	Antigen		
		Da-2(F)	Sr-3(F)	Sk-1(F)
Da-2	Da-2	+	−	−
	Sr-3	+	−	−
	Sk-1	+	−	−
Sr-3	Da-2	−	+	+
	Sr-3	−	+	+
	Sk-1	−	+	+
Sk-1	Da-2	−	+	+
	Sr-3	−	+	+
	Sk-1	−	+	+

(O): formalin-killed cells.
(F): heat-killed cells.

Recently, RFLP analysis and 16S rDNA sequencing have become popular in the taxonomy of bacteria.[12,13] Numerous isolates from environments can be rapidly grouped by RFLP analysis, which may be of important value for epidemiological purposes. This method would be a helpful tool to know the geographic distribution of *Vibrio* spp., the causative agents of sea urchin. On the

Table 9. Cross-agglutination reactions of anti-Da-2, Sr-3 and Sk-1 sera after absorption with cells of homologous and heterologous F-antigens

Antiserum	Absorbing antigen(F)	Antigen		
		Da-2(F)	Sr-3(F)	Sk-1(F)
Da-2	Da-2	−	−	−
	Sr-3	+	−	−
	Sk-1	+	−	−
Sr-3	Da-2	−	+	+
	Sr-3	−	−	−
	Sk-1	−	−	−
Sk-1	Da-2	−	+	+
	Sr-3	−	−	−
	Sk-1	−	−	−

(F): formalin-killed cells.

other hand 16S rDNA sequencing is an important tool to know the phylogenetic position of particular isolates. Besides, respiratory quinone component, whole cell proteins and ribotyping are also used to compare the closely related bacterial species. To clarify the taxonomic position of our strains further studies would be done by the above mentioned methods.

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