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RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF 16S rRNA GENE OF THE FAMILY VIBRIONACEAE ISOLATED FROM COLD MARINE ENVIRONMENTS

Hidetoshi URAKAWA, Kumiko KITA-TSUKAMOTO and Kouichi OHWADA

Ocean Research Institute, University of Tokyo, 15–1, Minamidai 1-chome, Nakano-ku, Tokyo 164-8639

Abstract: The present study was carried out for grouping and characterization of 60 natural isolates of psychrophilic and psychrotrophic vibrios isolated from deep-sea areas of the Western Pacific and Otsuchi Bay, where are located in northern Japan, using PCR-RFLP analysis of 16S rRNA genes

The 16S rRNA genes, about 1500 bp long, were amplified by PCR and used to determine the genotypes and phylogenetic affiliation of 60 natural isolates. Four tetrameric restriction enzymes (*Hha*I, *Dde*I, *Rsa*I and *Sau*3AI) were used for restriction fragment length polymorphism (RFLP) analysis. The 21 type and reference of the family Vibrionaceae were included in the analysis. A total of 81 strains were separated into 24 RFLP patterns. Five groups were obtained from the genetic distance tree and results showed significant differences in the genotype composition between the isolates from the deep-sea and Otsuchi Bay. We observed two large phylogenetic groups in psychrophilic and psychrotrophic vibrios. One was Group 5 (the *Vibrio marinus* group) which was distributed only in the depths of the Western Pacific. The other was Group 3 (the OC02 group) which was distributed in Otsuchi Bay.

key words: marine bacteria, Vibrionaceae, 16S rRNA, psychrophile, PCR-RFLP

Introduction

Members of the family Vibrionaceae are widely present in aquatic environments and are isolated frequently (AUSTIN *et al.*, 1979; SIMIDU *et al.*, 1980, 1982; HUQ and COLWELL, 1995). This family has been associated with fish and other poikilothermic animals, and includes several important pathogenic species to them (AUSTIN and AUSTIN, 1987), as well as to humans (BLAKE *et al.*, 1980). The genus *Vibrio* and *Photobacterium* are currently classified in the family Vibrionaceae together (BAUMANN and BAUMANN, 1984; BAUMANN *et al.*, 1984; FARMER III, 1992; FARMER III and HICKMAN-BRENNER, 1992; RUIMY *et al.*, 1994).

Marine psychrophilic (MORITA, 1975) and psychrotrophic (EDDY, 1960) gram-negative fermentative rods are found from the depths of the open ocean, polar regions and coastal water of high latitudes. They may contribute to the microbial biomass in the ocean, and cycling of matters within the psychrosphere. However, the number of known marine psychrophilic and psychrotrophic species has been limited, and

phylogenetic relationships of these organisms are still not clear.

Recently, 16S rDNA sequencing has become an important and powerful tool for taxonomical study. However, 16S rDNA sequencing cannot be considered appropriate for routine identification of multiple isolates from environments because it is time and cost consuming.

The number of studies based on small-subunit rRNA genes in combination with PCR and RFLP analysis has been increasing, because of their simplicity and rapidity (DOMENECH *et al.*, 1994; LAGUERRE *et al.*, 1994; HIRAISHI *et al.*, 1995).

The present study was carried out for grouping and genotyping of marine psychrophilic and psychrotrophic vibrios isolated from the Western Pacific and Otsuchi Bay using PCR-RFLP analysis of 16S rRNA genes.

Materials and Methods

Natural isolates

Twenty-nine strains from depths of the Pacific Ocean were collected on five cruises of research vessels, the HAKUHO-MARU and the TANSEI-MARU, Ocean Research Institute, University of Tokyo from 1991 to 1995 (Fig. 1 and Table 1). Water samples were

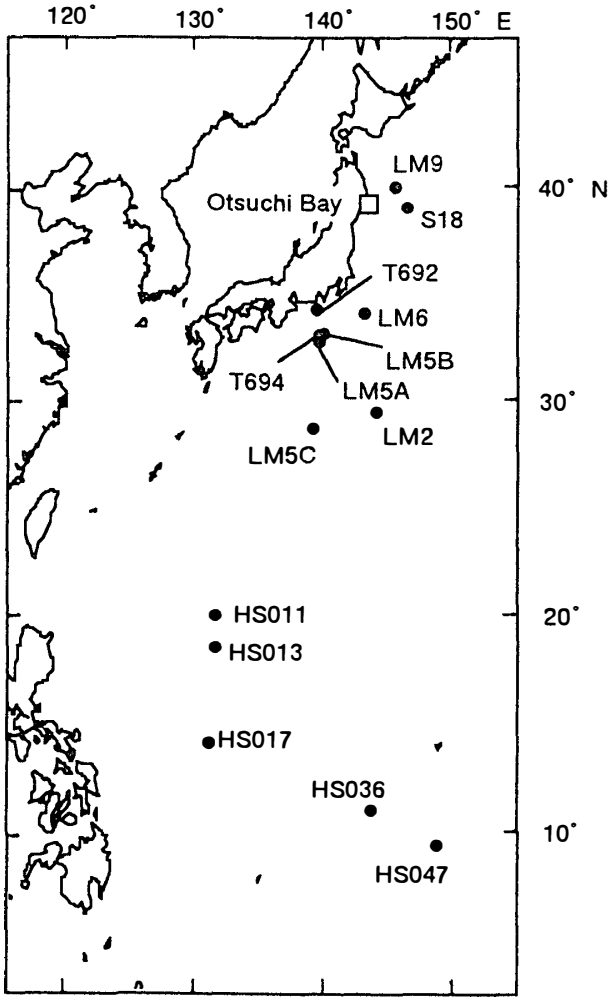

Fig. 1. Sampling locations of natural isolates.
Samplings of the open ocean were carried out during five cruises; KH95-2 (station code: HS series) (Aug. 1995), KT94-12 (T694) (July 1994), KH94-3 (LM series) (Oct. 1994), KT92-4 (T692) (July 1992) and KH91-6 (S18) (Nov. 1991).

Table 1. Natural isolates used in this study

16S rDNA genotype	Strain	Sampling location	Sampling depth (m)	Temperature (°C)	Isolated from:	Year/Month	Growth at: 4	20	30	35°C
1	OC02	Otsuchi Bay	0	5 3	Water	1996/MAR	+	+	−	−
1	OC25	Otsuchi Bay	3	5.4	Water	1996/MAR	+	+	−	−
1	OC26	Otsuchi Bay	5	5.6	Water	1996/MAR	+	+	−	−
1	OC44	Otsuchi Bay	1	5 1	Water	1996/MAR	+	+	−	−
1	OC45	Otsuchi Bay	1	5 1	Water	1996/MAR	+	+	+	−
1	OC47	Otsuchi Bay	2	5.1	Water	1996/MAR	+	+	−	−
1	OC50	Otsuchi Bay	2	5 1	Water	1996/MAR	+	+	−	−
1	OC71	Otsuchi Bay	20	6 2	Water	1996/MAR	+	+	−	−
1	OC74	Otsuchi Bay	22	6.3	Water	1996/MAR	+	+	−	−
1	OC88	Otsuchi Bay	1	5.1	Water	1996/MAR	+	+	−	−
1	OW21	Otsuchi Bay	0	6 0	Water	1994/MAR	+	+	+	−
1	OW22	Otsuchi Bay	0	6.0	Water	1994/MAR	+	+	+	−
1	OW23	Otsuchi Bay	0	6.0	Water	1994/MAR	+	+	+	−
1	OW38	Otsuchi Bay	0	6 7	Water	1994/MAR	+	+	+	−
2	OC11	Otsuchi Bay	1	5 4	Water	1996/MAR	+	+	−	−
2	OC17	Otsuchi Bay	2	5.4	Water	1996/MAR	+	+	−	−
2	OC33	Otsuchi Bay	8	5.8	Water	1996/MAR	+	+	−	−
2	OC102	Otsuchi Bay	10	5.8	Water	1996/MAR	+	+	+	−
2	OC116	Otsuchi Bay	0	4.7	Water	1996/MAR	+	+	−	−
2	OW31	Otsuchi Bay	0	6.7	Water	1994/MAR	+	+	+	−
2	OW41	Otsuchi Bay	0	6.9	Water	1994/MAR	+	+	+	−
3	OS55	Otsuchi Bay	0	ND[a]	Sediment	1994/MAR	+	+	+	−
4	OC77	Otsuchi Bay	22	6.3	Water	1996/MAR	+	+	−	−
4	OC84	Otsuchi Bay	1	5.1	Water	1996/MAR	+	+	−	−
4	OC117	Otsuchi Bay	0	4.7	Water	1996/MAR	+	+	−	−
4	HAR71	LM6	492	12 8	Water	1994/MAR	+	+	−	−
5	OC37	Otsuchi Bay	0	5.1	Water	1996/MAR	+	+	+	−
6	OW66	Otsuchi Bay	0	6.8	Water	1994/MAR	+	+	+	−
7	OS58	Otsuchi Bay	9	ND	Sediment	1994/MAR	+	+	+	−
8	HAR04	LM2	1001	3.9	Water	1994/OCT	+	+	−	−
8	HAR05	LM5A	3732	1.5	Water	1994/OCT	+	−	−	−
8	HAR08	LM2	1001	3 9	Water	1994/OCT	+	+	−	−
8	HAR10	LM5C	3628	1 5	Water	1994/OCT	+	−	−	−
8	HAR12	LM5C	3153	1.6	Water	1994/OCT	+	−	−	−
8	HAR13	LM5B	1488	2 6	Water	1994/OCT	+	−	−	−
8	HAR65	LM5C	2471	1.7	Water	1994/OCT	+	−	−	−
8	J13	T692	3600	ND	Water	1992/JUL	+	−	−	−
8	J28	S18	4063	1.4	Water	1991/NOV	+	−	−	−
8	J33	S18	4063	1.4	Water	1991/NOV	+	−	−	−
8	J35	S18	4063	1.4	Water	1991/NOV	+	−	−	−
8	ODA02	T694	500	ND	Water	1994/JUL	+	−	−	−
8	HAS1123	HS011	502	10.9	Water	1995/AUG	+	+	−	−
8	SC20	HS013	5998	1.8	Water	1995/AUG	+	+	−	−
8	SC22	HS036	8683	2 1	Water	1995/AUG	+	+	−	−
8	SC24	HS013	5000	1.6	Water	1995/AUG	+	+	−	−
8	SC25	HS017	6346	1.9	Water	1995/AUG	+	+	−	−
8	SC27	HS047	2946	1.6	Sediment	1995/AUG	+	+	−	−
9	HAR06	LM2	9706	2.1	Water	1994/OCT	+	−	−	−

Table 1. (Continued)

16S rDNA genotype	Strain	Sampling location	Sampling depth (m)	Temperature (°C)	Isolated from:	Year/Month	Growth at: 4	20	30	35°C
10	HAR70	LM6	492	12.8	Water	1994/OCT	+	+	−	−
10	HAR75	LM9	6496	1.7	Water	1994/OCT	+	+	+	+
11	OS53	Otsuchi Bay	9	ND	Sediment	1994/MAR	+	+	+	−
12	HAR19	LM2	3009	1.6	Water	1994/OCT	+	+	−	−
12	HAR23	LM2	3009	1.6	Water	1994/OCT	+	+	−	−
12	HAR73	LM6	183	18.6	Water	1994/OCT	+	+	+	−
12	SK29	HS036	6107	ND	Water	1995/AUG	+	+	+	−
12	SK30	HS036	6107	ND	Water	1995/AUG	+	+	+	−
13	HAR72	LM2	9706	2.1	Water	1994/OCT	+	+	+	−
13	HAR74	LM6	242	17.7	Water	1994/OCT	+	+	+	+
14	OW26	Otsuchi Bay	0	6.0	Water	1994/MAR	+	+	−	−
15	OM2	Otsuchi Bay	0	ND	Wood block	1994/MAR	+	+	+	−

[a] ND, No data.

collected using Niskin butterfly water samplers (General Oceanics, Florida, U.S.A.). Sediment samples were collected using a multiple core sampler (Rigosha Co. Ltd., Tokyo, Japan). Samples were stored on board the ship at 4°C. Half to 1/5 strength ZoBell 2216E agar plates or broth (ZoBell, 1946; Oppenheimer and ZoBell, 1952) were used for isolation.

Thirty-one Otsuchi Bay strains were isolated from water and sediment samples collected in March, 1994 and 1996 (Fig. 1 and Table 1). Water samples were collected using a Van Dorn water sampler (Rigosha Co. Ltd., Tokyo, Japan). Sediment samples were collected using a Smith-McIntyre sediment sampler (Rigosha Co. Ltd., Tokyo, Japan). Collected samples were immediately spreaded on half-strength ZoBell 2216E agar plates. The samples collected in 1994 and 1996 were incubated at 4°C and 15°C, respectively.

The bacterial isolates were purified by spreading on half-strength ZoBell 2216E agar plates. Gram-negative, Na^+-requiring, oxidase-positive and glucose fermentative rods were identified as members of the family Vibrionaceae. All isolates were able to grow at 4°C.

Type and reference strains

The 21 type and reference strains were also examined. Reference and type strains used in this study were as follows: *Vibrio alginolyticus* NCMB1903^T, *V. anguillarum* IFO13266^T, *V. campbellii* ATCC25920^T, *V. carchariae* ATCC35084^T, *V. fischeri* ATCC7744^T, *V. fluvialis* NCTC11327^T, *V. harveyi* ATCC14126^T, *V. logei* ATCC15382, *V. marinus* ATCC15381^T, *V. natriegens* CCM2575^T, *V. ordalii* ATCC35509^T, *V. parahaemolyticus* ATCC17802^T, *V. pelagius* ATCC25916^T, *V. salmonicida* ATCC43839^T, *V. splendidus* biovar I ATCC33125^T, *V. splendidus* biovar II ATCC33789, *V. vulnificus* ATCC27562^T, *Photobacterium angustum* ATCC25915^T, *P. damsela* ATCC35083, *P. leiognathi* ATCC25521^T, *P. phosphoreum* IAM12085.

Biochemical characterization tests

Most biochemical characterization tests were carried out at 20°C. The strains which did not grow above 20°C were cultured at 12–15°C. Gram reaction test was performed using 3% KOH solution (BUCK, 1982). The presence of cytochrome oxidase was tested with one or two day cultures on half-strength ZoBell 2216E medium, using filter papers with 1% n,n-dimethyl-p-phenylene-diamine hydrochloride (Kovacs oxidase test). Fermentative or oxidative utilization of glucose was tested by OF-basal medium (Difco Laboratories, Detroit, Michigan, U.S.A.) supplemented with 75% filtered and aged sea water. Growth at 4, 20, 30 and 35°C was examined with peptone water (1% peptone [Difco] supplemented with 3% NaCl). The Na^+-requirement was tested by using peptone water. Peptone water supplemented with 3% NaCl was used as a control. Cell morphology and motility were examined under a light microscope after growth in half-strength ZoBell 2216E broth.

DNA extraction

Cells were incubated at 20°C on half-strength ZoBell 2216E agar plates. *Vibrio marinus* and the isolates which did not grow above 20°C were cultured at 12–15°C. After harvest, cells were washed and suspended in sterile distilled water. For each strain, 20 μl of the suspension was mixed with 5 μl of proteinase K (1 mg/m*l*) and 25 μl of 2 × K buffer (40 mM Tris Buffer, 0.2% nonidet p-40, 0.2 mM EDTA, 1% Tween 20, distilled water, pH 8.0). The mixture was heat-treated at 60°C for 20 min, and at 100°C for 5 min, then cooled rapidly on ice and centrifuged at 8000 rpm for 5 min. The supernatant was transfered to a new plastic tube and used for PCR amplification.

PCR amplification

Primers 20 F (5′-AGAGTTTGATCCTGGCTCAG-3′, positions 8 to 27 of *Escherichia coli* 16S rRNA numbering) and 1500 R (5′-GGCTACCTTGTTACGACTT-3′, positions 1510 to 1492) described by WEISBURG *et al.* (1991) were used for PCR amplification. The oligonucleotides were synthesized by Sawady Technology Co., Tokyo. The PCR mixture consisted of 5 μl of reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], 1% TritonX-100), 3 μl of 25 mM $MgCl_2$, 5 μl of a mixture of the four deoxyribonucleotide 5′-triphosphates (25 μM each), 1 μl (10 pmol) of each PCR primer pair and 0.5 μl (2.5 U) of Taq DNA polymerase (Toyobo Co. Ltd., Osaka, Japan). Each sample was made up to 50 μl with distilled water. The mixture was covered with mineral oil (Pharmacia LKB Biotechnology, Uppsala, Sweden) before incubation. DNA amplification was done in a DNA thermal cycler (MiniCycler TM; MJ RESEARCH, Inc., Massachusetts, U.S.A.), with the following temperature profile: an initial denaturation at 94°C for 2 min; 25 cycles of denaturation (2 min at 94°C), annealing (1.5 min at 45°C), and extension (2 min at 72°C); and a final extension at 72°C for 3 min. Amplified DNA was examined by horizontal electrophoresis on a 1.0% agarose gel in TAE electrophoresis buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA) with 2 μl aliquots of PCR product.

RFLP analysis

The 6–8 μl of PCR products were digested with the four tetrameric restriction

enzymes (*Hha*I, *Dde*I, *Rsa*I and *Sau*3AI) (Toyobo) at 37°C for 60 min according to the manufacturer's instructions. These enzymes were chosen to obtain good numbers of fragments from the members of the family Vibrionaceae by using the GENETYX program (Software Development Co., Tokyo, Japan) and the published gene sequences of 16S rRNA gene. Restricted DNA was analyzed by horizontal electrophoresis on a 4% NuSieve 3 : 1 agarose gel (FMC BioProducts, Rockland, Maine, U.S.A.) in TAE electrophoresis buffer. Electrophoresis was carried out at 50 V for 150–170 min with a Mupid mini-gel electrophoresis apparatus (Advance Co., Tokyo, Japan) on ice. After electrophoresis, the gels were stained with aqueous solution of ethidium bromide. Scanning image analysis of the gel and estimation of fragment size were carried out with a densitograph imaging analyzer (Atto Co., Tokyo, Japan).

Informative bands were scored as to their presence or absence of two characters. Genetic distances were calculated by percentage divergence, and a genetic distance tree was constructed by the neighbor-joining method (SAITOU and NEI, 1987) using a Molecular Evolutionary Genetics Analysis (MEGA) software version 1.01 (KUMAR *et al.*, 1993).

Results

RFLP analysis

From 3 to 5 fragments were obtained with each of the four endonucleases. Good resolution to distinguish each cutting pattern was obtained using the Mupid mini-gel electrophoresis system on ice. Restriction fragments shorter than 99 bp produced by some endonucleases were not used for analysis. The numbers of restriction patterns obtained with each of the four tetrameric restriction enzymes were as follows: *Hha*I (5 patterns), *Dde*I (8 patterns), *Rsa*I (10 patterns), *Sau*3AI (6 patterns). Twenty-four different 16S rDNA genotypes were detected in the 81 strains analyzed by the PCR-RFLP method with the four tetrameric restriction enzymes (Table 2). In 24 different 16S rDNA genotypes, 15 different 16S rDNA genotypes were obtained from strains isolated from environmental samples. In 15 genotypes, 9 different 16S rDNA genotypes (genotype; 1, 2, 3, 5, 6, 7, 11, 14 and 15) consisted of strains isolated from Otsuchi Bay. On the other hand, 5 different 16S rDNA genotypes (genotype; 8, 9, 10, 12 and 13) were obtained only from isolates of the Pacific Ocean. Genotype 4 was the sole genotype including isolates both from coastal water of Japan and the Pacific Ocean (Table 2).

Growth tests

Capabilities of growth at 4, 20, 30 and 35°C of natural isolates were tested and listed in Table 1. Most isolates did not grow at 35°C (96.7%). The 66.7 and 16.7% strains did not grow at 30 and 20°C, respectively. All strains, which did not grow at 20°C, were isolates from the deep areas of the Pacific Ocean.

16S rDNA genotyping

Genotype 1 contained 14 strains isolated from Otsuchi Bay. This genotype was detected from samplings in both 1994 and 1996.

Genotype 2 was formed by 7 strains isolated from Otsuchi Bay. This genotype was also isolated from sampling in both 1994 and 1996.

Table 2. The 16S rDNA genotypes obtained by digestion with the four tetrameric restriction enzymes.

16S rDNA genotypes	No of strains	Restriction patterns of 16S rRNA genes digested with.				Reference strains	Natural isolates
		*Hha*I	*Dde*I	*Rsa*I	*Sau*3AI		
1	14	a	a	a	a		OC02, OC25, OC26, OC44, OC45, OC47, OC50, OC71, OC74, OC88, OW21, OW22, OW23, OW38
2	7	a	a	a	b		OC11, OC17, OC33, OC102, OC116, OW31, OW41
3	1	a	a	a	c		OS55
4	4	b	a	a	a		OC77, OC84, OC117, HAR71
5	1	b	a	a	b		OC37
6	1	b	a	a	c		OW66
7	1	a	a	b	d		OS58
8	18	a	b	c	c		HAR04, HAR05, HAR08, HAR10, HAR12, HAR13, HAR65, 13A, 28A, 33A, 35A, ODA02, HAS1123, SC20, SC22, SC24, SC25, SC27
9	2	a	c	c	c	*V marinus*	HAR06
10	2	a	d	c	e		HAR70, HAR75
11	2	a	e	c	e	*V. logei*	OS53
12	6	c	a	d	c	*P angustum*	HAR19, HAR23, HAR73, SK29, SK30
13	2	c	f	e	c		HAR72, HAR74
14	1	c	f	f	c		OW26
15	1	d	a	g	f		OM2
16	1	a	a	h	a	*V. splendidus* biotype I	
17	1	a	a	h	f	*V splendidus* biotype II	
18	1	a	e	1	e	*V salmonicida*	
19	2	c	a	e	c	*P leiognathi*	
						P phosphoreum	
20	1	c	a	j	d	*P. damsela*	
21	1	d	g	c	f	*V fischeri*	
22	8	e	a	h	f	*V alginolyticus*	
						V campbellii	
						V carchariae	
						V harveyi	
						V. natriegens	
						V parahaemolyticus	
						V pelagius	
						V vulnificus	
23	2	e	d	g	f	*V anguillarum*	
						V ordalii	
24	1	e	h	g	c	*V fluvialis*	

Genotype 3 was consisted of 1 strain, OS55 which was isolated from sediment of Otsuchi Bay in 1994.

Genotype 4 contained 4 strains, OC77, OC84, OC117 and HAR71. HAR71 was isolated from the 492 m depth of the Pacific Ocean. Three other strains were isolated from Otsuchi Bay.

Genotypes 5, 6, 7 contained 1 strain each from Otsuchi Bay. OC37 (genotype 5) and OW66 (genotype 6) were isolated from surface water of Otsuchi Bay in 1996 and in 1994, respectively. OS58 (genotype 7) was isolated from sediment of Otsuchi Bay in 1994.

Genotype 8 contained 18 strains; all members of this group were collected from the depths of the open ocean. It was the largest group in this study. The members of this genotype were collected during four cruises. Isolates were distributed among various sampling points and depths of the Pacific Ocean. None of the members grew above 30°C (Table 1).

Genotype 9 consisted of one deep sea isolate, HAR06 and psychrophilic type strain, *Vibrio marinus*. Genotype 8 and 9 showed the same restriction patterns with *Hha*I, *Rsa*I and *Sau*3AI digestions, but they showed different patterns when digested with *Dde*I. It is not clear whether this was due to species differences or intraspecies divergences.

Genotype 10 included 2 strains. Both isolates were collected from different sites and different depths. HAR70 was collected from LM6 (492 m) and HAR75 was collected from LM9 (6496 m).

Genotype 11 consisted of OS53 isolated from sediment of Otsuchi Bay in 1994. *V. logei* ATCC15382 was also included in this group.

Genotype 12 grouped 5 strains; it was the predominant genotype isolated from the open ocean. *P. angustum* ATCC25915^T was included in this group. Every member of this group was collected from various sites and depths of the Pacific Ocean.

Genotype 13 consisted of 2 strains. HAR72 was collected from LM2 (9706 m, 2.1°C) and HAR74 from LM6 (242 m, 17.7°C). Both isolates were collected from different sites and depths.

Genotypes 14, 15 consisted of 1 strain each isolated from Otsuchi Bay. OW26 (genotype14) was collected from surface water of Otsuchi Bay in 1994. OM2 (genotype 15) was isolated from a drifting wood block collected in front of Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo in 1994.

Genotypes 16 to 24 consisted of only type and reference strains (Table 2).

Phylogenetic relationships

To estimate the genetic relationships between PCR-amplified 16S rRNA genes, informative bands were scored as to their presence or absence. Fourty-seven informative characters were scored from the four endonucleases. A genetic distance matrix was constructed by percent divergence (Table 3). A genetic distance tree was constructed by the neighbor-joining method, and five groups were detected (Fig. 2).

Table 3 Genetic distances matrix among 16S rDNA genotypes based on the RFLP data set

Genotypes	Organisms	Genetic distance from :																						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	OC02		0.0185	0.0185	0.0556	0.0741	0.0741	0.2407	0.2222	0.2037	0.1667	0.2222	0.2037	0.2037	0.2778	0.2593	0.1481	0.2222	0.2222	0.1296	0.2593	0.2037	0.2778	0.3148	0.2407
2	OC11			0.0370	0.0741	0.0556	0.0926	0.2222	0.2407	0.2222	0.1481	0.2037	0.2222	0.2222	0.2963	0.2778	0.1667	0.2407	0.2037	0.1481	0.2407	0.2222	0.2963	0.3333	0.2593
3	OS55				0.0741	0.0926	0.0556	0.2222	0.2037	0.1852	0.1852	0.2407	0.1852	0.1852	0.2593	0.2407	0.1667	0.2037	0.2407	0.1111	0.2407	0.1852	0.2593	0.2963	0.2222
4	OC77					0.0185	0.0185	0.2963	0.2778	0.2593	0.2222	0.2778	0.2593	0.2593	0.3333	0.2778	0.2037	0.2778	0.2778	0.1852	0.3148	0.2222	0.2963	0.3333	0.2593
5	OC37						0.0370	0.2778	0.2963	0.2778	0.2037	0.2593	0.2778	0.2778	0.3519	0.2963	0.2222	0.2963	0.2593	0.2037	0.2963	0.2407	0.3148	0.3519	0.2778
6	OW66							0.2778	0.2593	0.2407	0.2407	0.2963	0.2407	0.2407	0.3148	0.2593	0.2222	0.2593	0.2963	0.1667	0.2963	0.2037	0.2778	0.3148	0.2407
7	OS58								0.3889	0.3704	0.2963	0.3519	0.2963	0.3704	0.3704	0.2778	0.2407	0.2037	0.3148	0.2963	0.1296	0.2963	0.2593	0.3333	0.3333
8	HAR05									0.0926	0.2407	0.2222	0.3519	0.2407	0.3148	0.4074	0.3333	0.3704	0.2963	0.2778	0.4074	0.2778	0.4259	0.3889	0.3148
9	HAR06										0.2222	0.2037	0.3333	0.2222	0.2963	0.3889	0.3148	0.3519	0.2778	0.2593	0.3889	0.2593	0.4074	0.3704	0.2963
10	HAR70											0.1296	0.3333	0.2963	0.3704	0.3519	0.2778	0.3148	0.2037	0.2593	0.3148	0.2222	0.3704	0.2963	0.3333
11	OS53												0.3889	0.2407	0.3148	0.4074	0.3333	0.3704	0.0741	0.3148	0.3704	0.2778	0.4259	0.4259	0.3519
12	HAR19													0.1481	0.1481	0.3148	0.2037	0.2407	0.3889	0.0741	0.2407	0.3333	0.2963	0.3704	0.2963
13	HAR72														0.0741	0.3889	0.3148	0.3519	0.2778	0.0741	0.2778	0.2963	0.4074	0.4074	0.2963
14	OW26															0.3519	0.2778	0.3148	0.3519	0.1481	0.3148	0.3704	0.3704	0.3704	0.2593
15	OM2																0.1852	0.1111	0.4444	0.3148	0.3333	0.1667	0.1296	0.1296	0.1667
16	*V. splendidus* biovar I																	0.0741	0.3333	0.2407	0.2963	0.3148	0.1296	0.2407	0.1667
17	*V. splendidus* biovar II																		0.3704	0.2778	0.2593	0.2407	0.0556	0.1667	0.2037
18	*V. salmonicida*																			0.3519	0.3333	0.3519	0.4259	0.4630	0.3889
19	*P. lelognathi*																				0.2037	0.2593	0.3333	0.3704	0.2963
20	*P. damsela*																					0.3148	0.315	0.389	0.389
21	*V. fischeri*																						0.259	0.259	0.296
22	*V. alginolyticus*																							0.111	0.148
23	*V. anguillarum*																								0.111
24	*V. fluvialis*																								

Genetic distances matrix were constructed by percent divergence The 16S rDNA genotypes (1 to 24) are defined in Table 2. Organisms are representatives of each 16S rDNA genotype

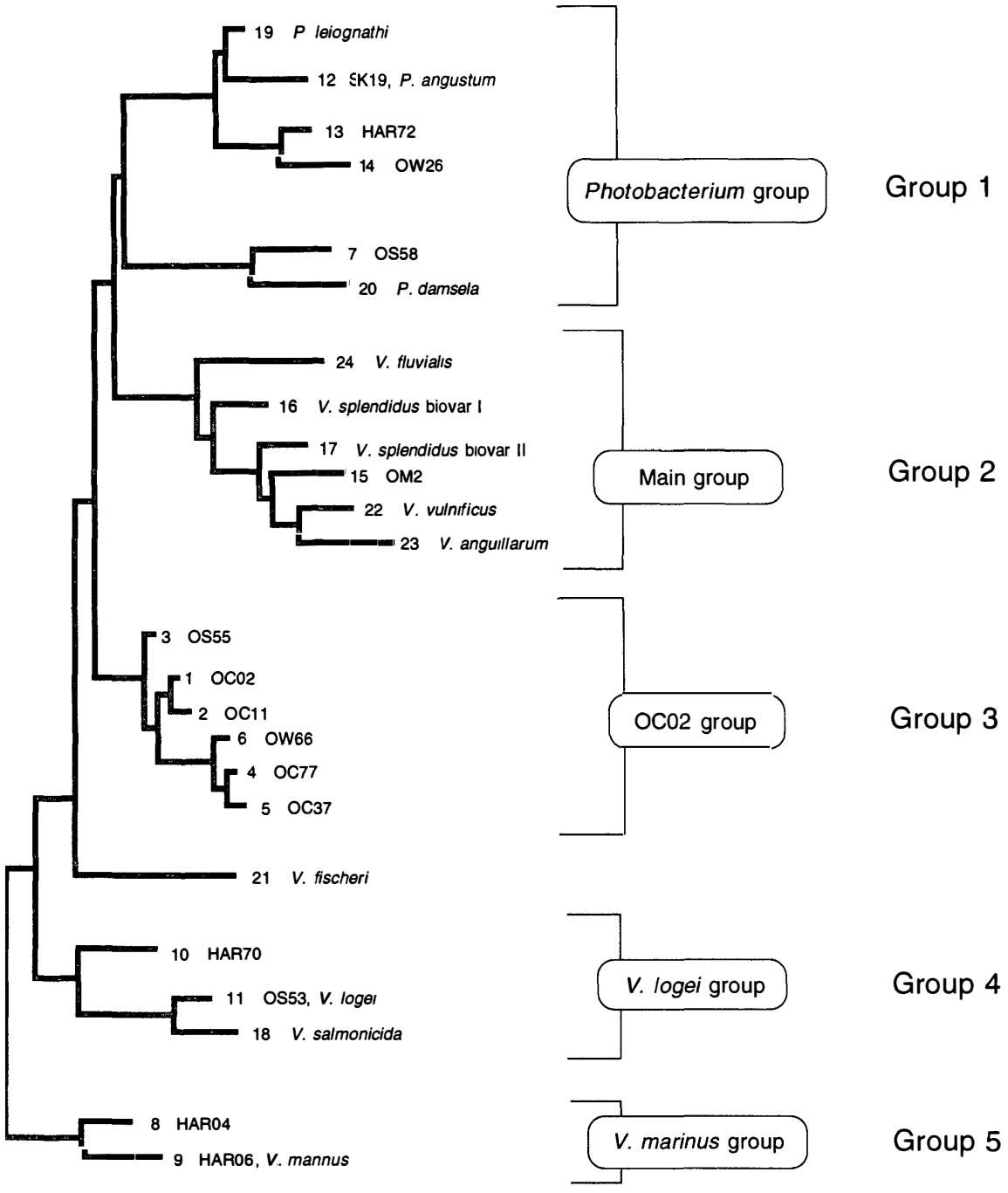

Fig. 2. Genetic distance tree based on the RFLP data set by the neighbor-joining method. Numbers indicate 16S rDNA genotypes (see Table 2). Organisms are representatives of each 16S rDNA genotype.

Discussion

The 16S rRNA sequence data will be needed to assess correct relationships between the strains belonging to this group and other known *Vibrio* species. PCR-RFLP analysis with the four restriction enzymes would be helpful to assess the approximate relationships among many isolates from environmental samples, before analysis of the 16S rRNA sequence.

To examine the phylogenetic affiliation of the numerous isolates from environments using PCR-RFLP analysis, determination of the minimum number set of the restriction enzymes will be needed. LAGUERRE *et al.* (1994) reported that the four restriction enzyme (4 base cutters) combination was the minimum set needed to discriminate strains used in their study. MOYER *et al.* (1996) reported that combination of the three or four tetrameric restriction enzymes gave good resolution results for the phylogeny of their computer-simulated bacterial groups.

In the present study, we checked the validity of our analysis and obtained a group by adding on the type and reference strains in the analysis (Fig. 2). Type and reference strains were grouped in accordance with 16S rRNA sequence data (KITA-TSUKAMOTO *et al.*, 1993; RUIMY *et al.*, 1994). In this study, RFLP analysis with four tetrameric restriction enzymes (*Hha*I, *Dde*I, *Rsa*I and *Sau*3AI) was successful in obtaining approximate phylogenetic relationships among environmental isolates belonging to the family Vibrionaceae.

Group 1 (*Photobacterium* group) consisted of members of the genus *Photobacterium* (Fig. 2). *P. angustum*, *P. damsela*, *P. leiognathi* and *P. phosphoreum* were also included in it. This group was comprised of two clusters. One included *P. damsela* and OS58, one of which was isolated from sediment of Otsuchi Bay. It could be distinguished from other clusters. Another cluster included three other *Photobacterium* species and eight environmental strains. One strain, OW26, was isolated from Otsuchi Bay, and 7 other strains were isolated from the open ocean. This group included open ocean isolates, coastal isolates and several reference strains. It was confirmed that *Photobacterium* species are distributed widely from the coastal environment to the open ocean. Genotype 12 included *P. angustum* and 5 isolates; no isolates were luminous. HAR19 and HAR23 did not grow at 30°C, but HAR73, SK29, and SK30 grew above 30°C. Genotype 13 grouped two strains, HAR72 and HAR74. HAR72 had luminous character and HAR74 did not have. HAR72 was collected from LM2 (9706 m, 2.1°C) and HAR74 was collected from LM6 (242 m, 17.7°C). This result indicated that the members of this genotype are distributed from shallow water to deep water in the open ocean.

Group 2 (Main group) consisted of almost mesophilic type cultures and only one natural isolate from Otsuchi Bay. Other natural isolates were not included in this group. This result implies the possibility that many psychrophilic and psychrotrophic vibrios isolated from cold marine environments would not belong to this group and could be included in any of several other groups of vibrios.

Genotype 1 was the large genotype and comprised the same group (Group 3) as genotype 2 (Fig. 2). All members of this group were isolated from Otsuchi Bay and they were separated phylogenetically from other groups. All the strains included in

Group 3 (OC02 group) were isolated from Otsuchi Bay. The genetic distance tree obtained from the RFLP data set clearly indicates that this group is completely included in the family Vibrionaceae and is distinguished from 4 other groups (Fig. 2). To determine the phylogenetic affiliation of the members of the OC02 group, further studies will be needed.

Group 4 (*V. logei* group) consisted of two reference strains (*V. logei* and *V. salmonicida*) and three natural isolates (OS53, HAR70 and HAR75). OS53, isolated from sediment of Otsuchi Bay, was included in the same genotype as *V. logei*. Genotype 10 included HAR70 and HAR75. Both strains were luminous. It has been difficult to distinguish the strains of the genus *Photobacterium* from the luminous *Vibrio* species with simple biochemical characterizations. However, this PCR-RFLP analysis with the four restriction enzymes made it possible to easily determine the clear phylogenetic position among the luminous isolates (HAR70, HAR75 and HAR72) (Fig. 2).

Group 5 (*V. marinus* group) consisted of two genotypes. All strains belonging to this group were isolated from the deep Pacific Ocean and they were psychrophilic bacteria (MORITA, 1975). A total of 65.5% strains isolated from the deep-sea areas were included in this group. Genotype 8 was the largest genotype in the present study. The 18 strains were included in this genotype and were collected from 11 sampling sites of the Pacific Ocean (Fig. 1). Genotype 9 consisted of one natural isolate (HAR06) and one type strain, *V. marinus*, which is known as a representative marine psychrophilic bacterium (COLWELL and MORITA, 1964; MORITA, 1975). The strains belonging to genotype 8 have close genotypical relationships to *V. marinus* (Fig. 2). The differences of phenotypic characteristics between HAR06 and *V. marinus* were observed in maximum growth temperature. *V. marinus* can grow at 20°C but HAR06 did not grow at 20°C or above. Group 5 isolates were collected from wide depths (between 500 m to 9706 m) and areas (Fig. 1) in the deep-sea. The growth characteristics of the members of group 5 match the definition of psychrophiles (MORITA, 1975). This result implies the possibility that the closely related marine psychrophiles belonging to this group may be distributed over broad areas in the depths of the open ocean. A previous report of isolation supports our conclusion (COLWELL and MORITA, 1964). MACDONELL and COLWELL (1984) reported that on the basis of 5S rRNA sequence data, *V. marinus* showed little relation to other species of *Vibrio* and *Photobacterium*. From this result, *V. marinus* was proposed as a type species of a new genus "*Moritella*" (STEVEN, 1990). Partial and full 16S rRNA sequences also supported this proposal (GAUTHIER *et al.*, 1995; KITA-TSUKAMOTO *et al.*, 1993). However, the nomenclature "*Moritella marinus*" is still invalid. More detailed studies for our psychrophilic strains will be helpful to evaluate the new taxonomical status of *V. marinus*.

In summary, the PCR-RFLP method used in this study contributes to simple and rapid genotyping and classification among natural isolates belonging to the family Vibrionaceae. As a result of our study, significant differences were observed in the genotype composition between isolates from the deep-sea and Otsuchi Bay. We observed two phylogenetic groups in psychrophilic and psychrotrophic vibrios. One is the *V. marinus* group, which is distributed in the depths of the Pacific Ocean. The other is the OC02 group, which is distributed in Otsuchi Bay in winter.

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