The Journal of Microbiology, December 2003, p.312-319 Vol. 41, No. 4

Genotyping of Six Pathogenic *Vibrio* Species Based on RFLP of 16S rDNAs for Rapid Identification

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(Received June, 2003 / Accepted September 15, 2003)

In an attempt to develop a method for rapid and accurate identification of six *Vibrio* species that are clinically important and most frequently detected in Korea, 16S rDNA restriction fragment length polymorphism (RFLP) of *Vibrio* type strains, as well as environmental isolates obtained from the Korean coastal area, was analyzed using ten restriction endonucleases. Digestion of the 16S rDNA fragments amplified by polymerase chain reaction (PCR) with the enzymes gave rise to 2~6 restriction patterns for each digestion for 47 *Vibrio* strains and isolates. An additional 2~3 restriction patterns were observed for five reference species, including *Escherichia coli*, *Aeromonas hydrophila*, *A. salmonicida*, *Photobacterium phosphoreum*, and *Plesiomonas shigelloides*. A genetic distance tree based on RFLP of the bacterial species correlated well with that based on 16S rDNA sequences. The very small 16S rDNA sequence difference (0.1%) between *V. alginolyticus* and *V. parahaemolyticus* was resolved clearly by RFLP with a genetic distance of more than 2%. RFLP variation within a species was also detected in the cases of *V. parahaemolyticus*, *V. proteolyticus*, and *V. vulnificus*. According to the RFLP analysis, six *Vibrio* and five reference species were assigned to 12 genotypes. Using three restriction endonucleases to analyze RFLP proved sufficient to identify the six pathogenic *Vibrio* species.

Key words: *Vibrio*, pathogenic, genotyping, RFLP, identification

The family *Vibrionaceae* forms one of the most important bacterial groups among marine flora and comprises the *Vibrio*, *Photobacterium*, *Aeromonas*, and *Plesiomonas* species (Simidu *et al.*, 1977). These species are found most frequently in seawater, plankton, and the intestines of marine animals. According to studies carried out in the West Pacific, 80% of the bacteria found at the surface of seawater were *Vibrio* species (Simidu *et al.*, 1980). Some members of the family Vibrionaceae are pathogenic to humans and animals. In particular, the *Vibrio* species of *V. cholerae*, *V. alginolyticus*, *V. fluvialis*, *V. mimicus*, *V. parahaemolyticus*, and *V. vulnificus* are classified as clinically important human pathogens, since they cause diseases such as cholera, septicemia, and gastroenteritis (Östling *et al.*, 1980). *V. alginolyticus*, *V. harveyi*, *V. vulnificus*, and *V. proteolyticus* are known as fish (and sometimes marine invertebrate) pathogens. During the summer, when water temperatures rise above 20°C, *Vibrio* species grow quickly in seawater and the intestines of marine animals, thereby becoming a main pathway of human infection via ingestion of raw seafood or through wounds.

Rapid and accurate identification of these *Vibrio* species based on their physiological and biochemical properties has not been satisfactory, not only because the tests are laborious and time-consuming, but also because of inaccuracy due to the biochemical diversity of *Vibrio* isolates from various environmental samples. There are as many as 35 *Vibrio* species listed in Bergey's Manual (Holt *et al.*, 1994), but the identification key is variable according to different researchers (Bryant *et al.*, 1986; West *et al.*, 1986; Austin *et al.*, 1992; Alsina and Blanch, 1994). Previous studies on the diversity of *Vibrio* around the Korean coastal area using various physiological and biochemical tests revealed that *V. parahaemolyticus* and *V. alginolyticus*, pathogens causing gastroenteritis, were the most frequently detected *Vibrio* species (16% each) (Yoon *et al.*, 1996). Other pathogens frequently detected from the area included *V. fluvialis* (12%), *V. vulnificus* (6%), and *V. proteolyticus* (5%). However, a great range of biochemical variation was observed in most species of natural isolates (Yoon *et al.*, 1996).

Nucleotide sequence of 16S rDNA has been widely used to understand phylogenetic relationships among prokaryotes (Barry *et al.*, 1990; Weisburg *et al.*, 1991). Graham *et al.* (1991) applied phylogenetic relationships to species-specific identification of bacteria using hybridization between DNA and 16S rRNA, and aligning the nucleotide sequences of 16S rDNA. Phylogenetic relationships of various *Vibrio* species have also been characterized in detail using the sequences of 16S rDNA (Dorsch *et al.*, 1992; Kita-Tsukamoto *et al.*, 1993). How-

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ever, these processes are inappropriate for routine identification of bacterial strains due to the length of time required. Therefore, restriction fragment length polymorphism (RFLP), using 16S rDNA genes amplified by polymerase chain reaction (PCR), has been introduced for rapid and accurate identification of bacteria at the species level (Ralph *et al.*, 1993; Choudhury *et al.*, 1994; Moyer *et al.*, 1994; Urakawa *et al.*, 1999). The randomly amplified polymorphic DNA (RAPD) PCR method, developed for use in detecting *V. vulnificus,* also clearly differentiated this *Vibrio* species from the other members of the genus *Vibrio* (Warner and Oliver, 1999).

In this study, 16S rDNA of six *Vibrio* species that were known to be clinically important and/or detected most frequently in the Korean coastal area (Yoon *et al.*, 1996) were amplified by PCR and subjected to RFLP analysis using ten restriction endonucleases in an effort to develop a method that could be applied for rapid and accurate identification of *Vibrio* isolates. RFLPs of both *Vibrio* type strains and environmental isolates were determined for this purpose. A phylogenetic tree of the *Vibrio* species was established based on the RFLP pattern to verify its relevance to genetic fingerprinting.

Materials and Methods

Bacterial strains and medium

The *Vibrio* strains and isolates as well as those closely related bacteria that were used in this study are listed in Table 1. All the *Vibrio* isolates were obtained from various regions of the Korean coastal area and from marine animals as described previously (Yoon *et al.*, 1996). The *Vibrio* strains were cultured at 30°C in Luria-Bertani (LB) broth [1.0% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 3.0% (w/v) NaCl] with shaking (200 rpm), or on thiosulfate citrate bile salt sucrose agar plates [TCBS; 0.5% (w/v)

Table 1. Bacterial strains used in this study

Taxon	Strains	Source[a]
V. alginolyticus	ATCC 17749	ATCC
	IKHSM3-1, IS_1SM1, 3, 8	Sea mud
	CHSW1, ISSW4, IS_1SW4	Sea water
	IKHCM2-1, IKHTS2-2	Clam, shell
	IYCBA4	Sea bass
V. cholerae	ATCC 25872, IKHSM6, 10, IKHSW5, 9	NIH, Seamud, Seawater
V. fluvialis	ATCC 33809	ATCC
	IYCSW5	Sea water
V. parahaemolyticus I[b]	ATCC 17802	ATCC
	IKHSM3-3, ISSM3	Sea mud
	IYCSW3, 6	Sea water
V. parahaemolyticus II[b]	ATCC 33844	ATCC
	CHDSW5, CSYSW3, IKHSW2-1, 2-2	Sea water
	IYSW11, YCSM2	Sea mud
	IYCCM5	Clam
V. proteolyticus I[b]	ATCC 15338	ATCC
	IYSM 8	Sea mud
	IS_1SW 6	Sea water
V. proteolyticus II[b]	IS_1SW 7	Sea water
V. vulnificus I[b]	ATCC 27562, 29306	ATCC
	WDOS61, YKCB13	Oyster and Crab
V. vulnificus II[b]	ATCC 29307, CDC C7184	ATCC,CNNU
	CHUSW24, ISCSW3-2	Sea water
	MPRF33	Rock fish
	KGCB25	Crab and Clam
	SAOS38	Oyster
	KGSO22	Octopus
A. hydrophila	ATCC 7966	IMSNU
A. salmonicida	IFO 13896	IMSNU
Pl. shigelloides	IMSNU 10187	IMSNU
P. phosphoreum	IMSNU 12026	IMSNU
E. coli	ATCC 11775	IMSNU

[a]ATCC, American Type Culture Collection; CDC, Centers for Disease Control; CNNU, Chonnam National University, Kwangju, Korea; IFO, Institute for Fermentation; IMSNU, Institute of Microbiology, Seoul National University, Seoul, Korea; NIH, National Institute of Health, Seoul, Korea. All isolates were isolated from various regions of the Korean coastal area and from marine animals as described previously (Yoon *et al.*, 1996).
[b]The *Vibrio* strains and isolates were grouped based on the RFLP analysis in this study.

Bacto yeast extract, 1.0% (w/v) Bacto proteose peptone No. 3, 1.0% (w/v) sodium citrate, 1.0% (w/v) sodium thiosulfate, 0.8% (w/v) Bacto oxgall, 2.0% (w/v) Bacto saccharose, 1.0% (w/v) sodium chloride, 0.1% (w/v) ferric citrate, 0.04% (w/v) Bacto brom thymol blue, 1.5% (w/v) Bacto agar; Difco Laboratories Co., U.S.A.] (Lotz *et al.*, 1983). Other bacterial strains were cultured in LB broth containing 0.5% NaCl with shaking (200 rpm) at 37°C.

Chromosomal DNA extraction

Chromosomal DNA of bacteria was purified by the spooling method (Sambrook *et al.*, 1989) from 50 ml of overnight culture or was extracted by a freezing-thawing method. For the freezing-thawing method, overnight bacterial culture (1.5 ml) was harvested in a microcentrifuge (10,000 rpm, for 2 min) and resuspended in 100 μl of sterile distilled water. The cells were frozen and thawed 3~4 times and the supernatant was removed after centrifugation at 12,000 rpm for 5 min. The pellet was resuspended in 20 μl of sterile distilled water.

Polymerase chain reaction (PCR)

PCR was carried out according to Shirai *et al.* (1991) with some modifications. The PCR mixture consisted of 1~100 ng/μl of chromosomal DNA as template, 200 μM of dNTPs (Perkin-Elmer Cetus, USA), 25 mM of $MgCl_2$, 0.4 μM of each primer (universal primers; fD2, 5'-AGAGTTTGATC-ATGGCTCAG-3'; rP1, 5'-ACGGTTACCTTGTTACGACT T-3'; Bioneer Co., Korea), and Taq polymerase (Perkin-Elmer Cetus, USA; 2.5~5.0 Units) in 50 μl of reaction buffer [50 mM KCl, 100 mM Tris-HCl (pH 8.0)]. PCR was carried out using a GeneAmp PCR System 2400 (Perkin-Elmer Cetus, USA) as follows: an initial denaturation at 94°C for 5 min, 40 cycles of denaturation (94°C, 1 min), annealing (65°C, 1.5 min), extension (72°C, 2 min), and a final extension at 72°C for 5 min. Amplified DNA was examined by electrophoresis on a 0.8% agarose gel in TBE buffer [89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA (pH 8.5)].

Restriction fragment analysis

16S rDNA fragments amplified by PCR were digested using 10 restriction endonucleases (*Alu*I, *Hae*III, *Hha*I, *Hin*fI, *Hsp*92II, *Mbo*I, *Msp*I, *Pst*I, *Rsa*I, and *Taq*I; BRL, USA) individually, and according to the manufacturer's instructions. These enzymes were chosen based on the sequences of the recognition sites to obtain a good number of fragments from the *Vibrio* 16S rDNAs. Digestions using the enzymes were carried out at 37°C for 1~2 h, except in the case of digestions using *Taq*I, which were carried out at 65°C. Resulting restriction patterns were analyzed using 2.0 (w/v) agarose gel electrophoresis. RFLPs of the type strains, including *Vibrio* and related bacterial species, were also predicted using the sequences of 16S rDNA available in the DNA database (GenBank, USA) and DNASIS program (IBI, USA).

Construction of a genetic distance tree

A similarity matrix of the *Vibrio* species and the related bacterial 16S rDNA was constructed by the neighbor-joining method (Saitou and Nei, 1987). Informative DNA fragments, derived from restriction endonuclease digestion and longer than 100 bp, were scored for their presence or absence, and similarity and divergence were calculated. The genetic distance trees were constructed based on the RFLP data or the 16S rDNA sequence similarity using the numerical taxonomy system of a multivariate statistical program (NTSYS-pc, Exeter Software, USA).

Results and Discussion

Predicted 16S rDNA RFLP of the Vibrio and related bacterial strains

16S rDNA nucleotide sequences of the *Vibrio* and related bacterial type strains were obtained from the database and their RFLPs, generated using ten restriction endonucleases, were predicted. The ten restriction endonucleases employed in this study included eight tetrameric, one pentameric (*Hin*fI), and one hexameric (*Pst*I) restriction enzymes. Similarity of 16S rDNA among the *Vibrio* strains tested ranged from 83 to 99.9% (Table 2). Other bacterial strains of the family *Vibrionaceae* had 16S rDNA sequences that were 73.3-92.0% homologous to

Table 2. Similarity matrix of the *Vibrio* and related bacterial 16S rDNA sequences

Bacterial Strains	Identity (%)										
	1	2	3	4	5	6	7	8	9	10	11
1. *E. coli*	100.0										
2. *V. alginolyticus*	74.0	100.0									
3. *V. cholerae*	83.3	83.3	100.0								
4. *V. fluvialis*	73.0	89.0	83.7	100.0							
5. *V. parahaemolyticus*	73.3	99.0	83.3	93.0	100.0						
6. *V. proteolyticus*	76.7	96.3	85.3	94.3	95.7	100.0					
7. *V. vulnificus*	87.0	84.0	94.0	83.0	84.3	86.3	100.0				
8. *A. hydrophils*	84.0	81.3	88.7	73.3	78.3	79.3	86.3	100.0			
9. *A. salmonicida*	84.0	81.3	88.0	78.0	78.3	79.0	86.0	99.0	100.0		
10. *Pl. shigelloides*	77.0	90.7	83.7	89.7	91.3	92.0	82.3	76.3	83.7	100.0	
11. *P. phosphoreum*	84.0	83.0	89.7	81.0	82.0	83.7	89.0	92.6	93.0	87.7	100.0

those of the *Vibrio* strains, while a reference strain, *E. coli* ATCC11775, had a 16S rDNA sequence that was 73~87% similar to those of the *Vibrio* strains. *E. coli* 16S rDNA was 76~84% similar to those of other bacterial strains in the family Vibrionaceae.

Since end points of each sequence were variable, and detection of restriction fragments shorter than 100 bp from 2% agarose gels would be inaccurate, these fragments were excluded when comparing RFLP. Predicted RFLPs for ten *Vibrio* and five other bacterial 16S rDNAs based on ten restriction endonuclease recognition sites gave rise to a total of 58 different patterns. Genetic distance based on RFLPs of the bacterial strains ranged from 70~100% (Fig. 1), which correlated well with the 16S rDNA similarity. RFLPs of the *Vibrio* strains obtained from culture collection centers exhibited 84~100% similarity. The most striking difference between 16S rDNA similarity and RFLP was observed among *A. hydrophilia*, *A. salmonicida*, and *Pl. shigelloides*. Neither the 1.0% difference between *A. hydrophilia* and *A. salmonicida* 16S rDNA sequences nor the 23.7% difference between *A. hydrophilia* and *Pl. shigelloides* was detected by RFLP. However, the 7.0~16.3% differences among these bacteria and *P. phosphoreum* were clearly detected by RFLP. The difference between *E. coli* and other bacterial 16S rDNAs (73~87%) was resolved well by RFLP using the ten restriction endonucleases. From the analyses, RFLP of the *Vibrio* species using the enzymes was likely to be relevant to genotyping of the genus.

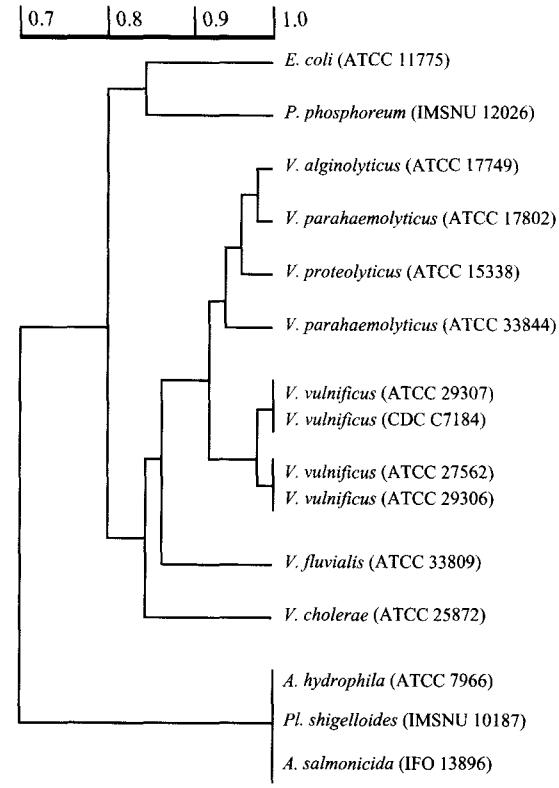

Fig. 1. A genetic distance tree based on predicted RFLPs of 16S rDNA of the *Vibrio* and reference species.

Amplification of the 16S rDNA

Optimal concentration of template DNA was determined to be in the range of 0.5~100 μg per reaction. Chromosomal DNA that was prepared by the spooling method, including phenol-chloroform extraction, produced a sufficient amount of PCR product, in the range of 0.5~10 μg per reaction (data not shown). On the other hand, 1 μg of chromosomal DNA per reaction was most appropriate for the DNA samples prepared by the freezing-thawing method (data not shown). PCR products produced by the two methods did not exhibit any difference at the background level or in digestion efficiency by various restriction endonucleases. In this study, the latter method was chosen for efficient and rapid amplification of 16S rDNA from various bacterial cells since this method took much

Table 3. Genotypes of the *Vibrio* and related bacterial strains based on RFLP using ten restriction endonucleases

Bacteria \ Enzyme	*Alu*I	*Hae*III	*Hha*I	*Hin*fI	*Hsp*92II	*Mbo*I	*Msp*I	*Pst*I	*Rsa*I	*Taq*I	Genotype[a]
V. alginolyticus	1	1	1	1	1	1	1	1	1	1	1
V. cholerae	4	1	2	3	2	2	4	1	2	2	2
V. fluvialis	4	3	1	1	4	3	3	2	3	3	3
V. parahaemolyticus I	1	2	1	1	3	1	2	1	1	1	4
V. parahaemolyticus II	6	2	1	1	3	1	2	1	1	1	5
V. proteolyticus I	3	3	1	1	2	1	3	1	1	1	6
V. proteolyticus II	1	2	1	1	2	1	3	1	1	1	7
V. vulnificus I	5	4	3	2	4	1	3	1	1	3	8
V. vulnificus II	2	4	1	1	5	1	3	1	4	4	9
A. hydrophila	7	5	4	4	6	4	5	3	5	3	10
A. salmonicida	7	5	4	4	6	4	5	3	5	3	10
P. phosphoreum	9	7	5	5	8	3	7	1	6	3	11
Pl. shigelloides	7	5	4	4	6	4	5	3	5	3	10
E. coli	8	6	5	5	7	1	6	1	6	4	12

[a]The 16S rDNA genotypes based on the combination of RFLPs obtained using ten restriction endonucleases.

less time and cost less. All chromosomal DNAs of the 47 *Vibrio* and five other bacterial strains and isolates that were analyzed in this study gave rise to a single 1.5 kb DNA fragment that corresponded to the predicted size of the 16S rDNA genes.

16S rDNA genotyping of the Vibrio strains

Digestion of the amplified 16S rDNA fragments by ten restriction endonucleases gave rise to 2~6 restriction patterns for the ten *Vibrio* strains and 37 *Vibrio* natural isolates from the Korean coastal area. Each unique restriction pattern consisted of 2~6 clearly distinct DNA bands. An additional 2~3 restriction patterns were observed for the five reference species, including *E. coli*, *A. hydrophila*, *A. salmonicida*, *P. phosphoreum*, and *Pl. shigelloides*, which were used to test the specificity of RFLPs in identifying *Vibrio* species. Among the enzymes used, *Alu*I produced the most diverse restriction patterns for the bacterial strains and isolates tested, followed by *Hsp*92II. In most of the cases, RFLP of the amplified 16S rDNA matched well to the predicted one. As the results show, nine different 16S rDNA genotypes for six *Vibrio* species and three for five reference species were derived from the restriction patterns (Table 3). A genetic distance tree based on the RFLP data was very similar to the similarity matrix of 16S rDNA sequences and fit well with the tree established based on the predicted data. Genetic distance among the *Vibrio* species was in the range of 79~100% (Fig. 2). In the case of *V. alginolyticus* and *V. parahaemolyticus*, 0.1% sequence differences between the species were detected clearly by RFLP with a genetic distance of 2~7%. *V. proteolyticus*, with a 4% sequence difference, showed 4~7% genetic distance from the two *Vibrio* species. The *V. vulnificus* group had 12% genetic distance from the above three *Vibrio* species, which were 14~17% different from them at the sequence level. *V. fluvialis* and *V. cholerae* were grouped farthest from the rest of the *Vibrio* species with 18% and 20% distance, respectively. Their sequence difference from the rest of the *Vibrio* species was 6~17%. *V. fluvialis* was unique among the *Vibrio* species tested in having a restriction site for *Pst*I, a hexameric restriction endonuclease. These data suggest that RFLPs of 16S rDNA have potential as a tool for rapid and accurate identification of these pathogenic *Vibrio* isolates at the species level, clearly distinguishing them from other members of the family *Vibrionaceae* and *E. coli*.

RFLP variations in a Vibrio species

Among the *Vibrio* strains analyzed, 11 *V. alginolyticus* strains and five *V. cholerae* strains showed a single unique RFLP for each species (Fig. 2). On the other hand, variation in RFLP among strains of a defined species was observed for *V. parahaemolyticus*, *V. proteolyticus*, and *V. vulnificus* species (Fig. 2 & Table 3). *V. parahaemolyticus* ATCC 33844, an isolate from a gastroenteritis patient, showed a restriction pattern different from that of the type strain, *V. parahaemolyticus* ATCC 17802, upon *Alu*I digestion. Two of the natural isolates showed the same RFLP as the latter, while nine natural isolates had the same pattern as the former. Genetic distance based on the RFLP using ten restriction endonucleases indicated that the former group

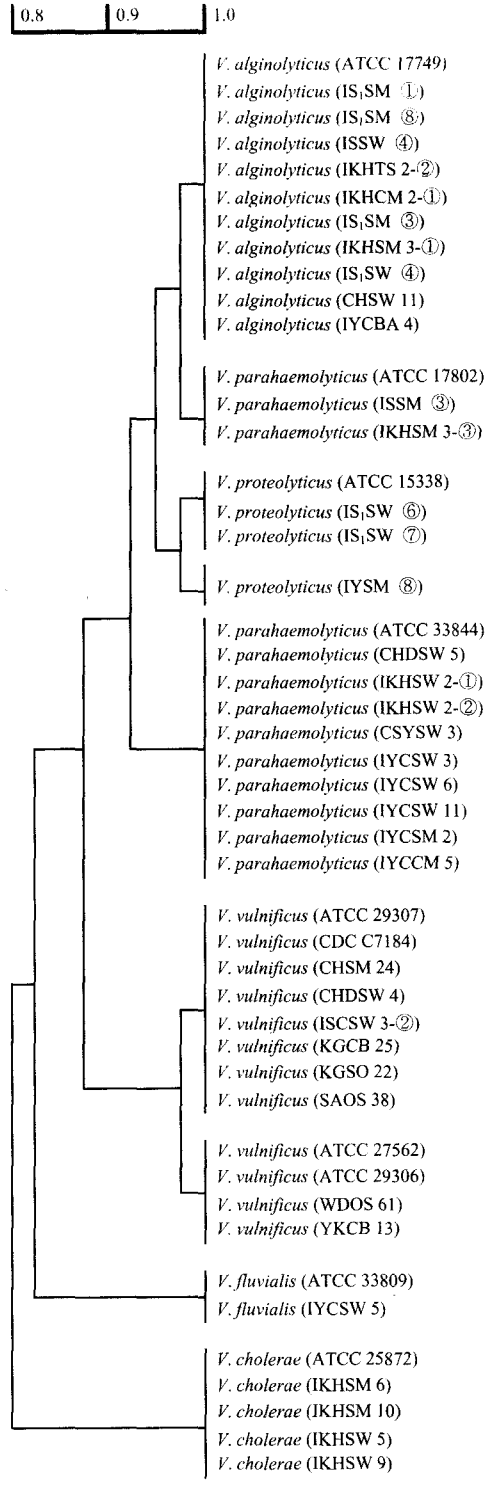

Fig. 2. A genetic distance tree of the *Vibrio* strains including natural isolates based on 16S rDNA RFLP similarity indices.

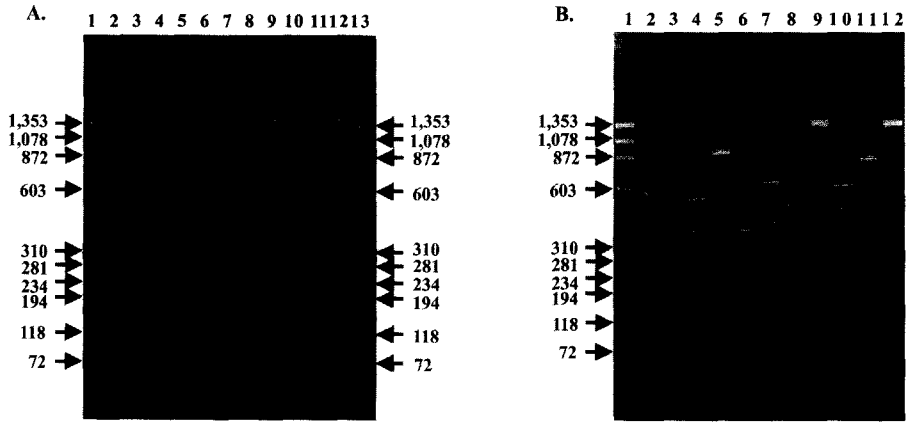

Fig. 3. RFLP of 16S rDNA amplified from *V. vulnificus* ATCC 27562 (A) and CDC C7184 (B). Lanes 1 and 13 were loaded with ΦX174 DNA/*Hae*III markers; lane 2, *Alu*I digestion; lane 3, *Hae*III digestion; lane 4, *Hha*I digestion; lane 5, *Hinf*I digestion; lane 6, *Hsp*92II digestion; lane 7, *Mbo*I digestion; lane 8, *Msp*I digestion; lane 9, *Pst*I digestion; lane 10, *Rsa*I digestion; lane 11, *Taq*I digestion; lane 12, no restriction.

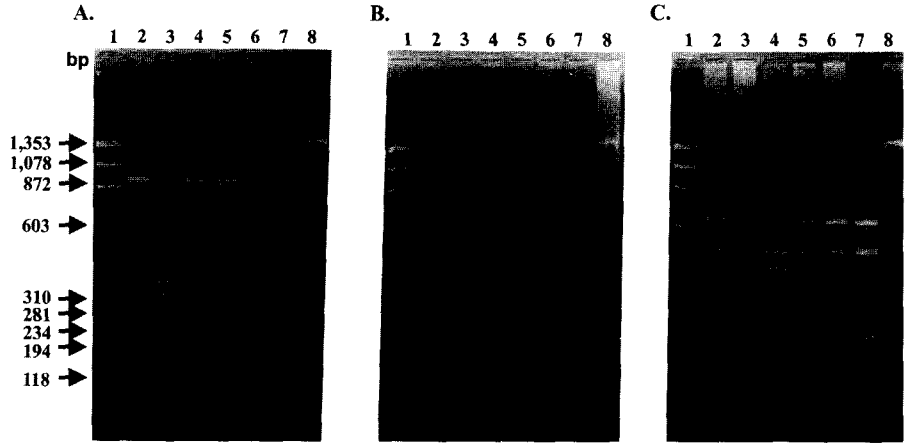

Fig. 4. RFLP of 16S rDNA amplified from six *Vibrio* species using three restriction endonucleases. Panel A represents RFLP generated by *Hinf*I digestion; panel B, *Hsp*92II digestion; and panel C, *Rsa*I digestion. Lane 1 were loaded with a marker, ΦX174 DNA digested with *Hae*III; lane 2, 16S rDNA of *V. alginolyticus* ATCC 17749; lane 3, *V. cholerae* ATCC 25872; lane 4, *V. fluvialis* ATCC 33809; lane 5, *V. parahaemolyticus* ATCC 17082; lane 6, *V. proteolyticus* ATCC 15338; lane 7, *V. vulnificus* ATCC 27562; lane 8, no restriction.

diverged from the latter group further than from *V. proteolyticus* species. A group of *V. vulnificus* strains (ATCC 29307 and CDC C7184) also showed variation in RFLP from the type strain group (*V. vulnificus* ATCC 27562 and ATCC 29306). The two groups differed from each other in five restriction patterns, including those generated by *Alu*I, *Hae*III, *Hinf*I, *Hsp*92II, and *Msp*I (Fig. 3). However, these two groups were genetically closer to each other than to any other *Vibrio* species. Warner and Oliver (1999) reported that *V. vulnificus* species were genetically quite heterogeneous based on RAPD analysis. In the case of *V. proteolyticus*, two genotypes were observed based on RFLPs; one for the type strain (ATCC15338) and a couple of natural isolates, the other for a strain isolated from seawater (IS_1SW7). The two groups were genetically closer to each other than to other *Vibrio* species. These results indicate that RFLP of 16S rDNA is useful in detecting genetic variation occurring within a species. They also suggest that intraspecific relationships might need to be considered when interpreting RFLP analysis of 16S rDNA genes (Navarro *et al.*, 1992; Park *et al.*, 2003).

Identification of the Vibrio strains using three restriction endonucleases

From the results obtained in this study, RFLP using three restriction endonucleases turned out to be enough for rapid identification of the *Vibrio* species. Three restriction endonucleases including *Hinf*I, *Hsp*92II, and *Rsa*I gave rise to 17 signature restriction fragments and a total of nine genotypes among six pathogenic *Vibrio* species (Fig. 4; Table 4). *V. parahaemolyticus* group II exhibited one restriction fragment different from those of *V. parahaemolyticus* group I upon *Rsa*I digestion, while *V. proteolyticus* group II and *V. vulnificus* group II also exhibited one restriction fragment different from those *of V. proteolyticus* group I and *V. vulnificus* group I, respectively, upon *Hinf*I digestion. The differences made it possible to differentiate the subgroups (Table 3).

E. coli and other members of the family Vibrionaceae showed RFLPs that could differentiate them clearly from the *Vibrio* species. Urakawa *et al.* (1999) analyzed RFLPs of 31 *Vibrio* species using five restriction enzymes and grouped them into 24 genotypes. According to their analysis,

Table 4. Signature restriction fragments and genotypes generated from 16S rDNA using three restriction endonucleases

Bacteria \ Enzyme	*Hinf*I		*Hsp*92II		*Rsa*I	
V. alginolyticus	942, 353, 180 bp	(1)[a]	370, 218 bp	(1)	673, 503 bp	(1)
V. parahaemolyticus I	942, 353, 180 bp	(1)	370, 218, 180 bp	(2)	673, 503 bp	(1)
V. parahaemolyticus II	942, 353, 180 bp		370, 218, 180 bp		673, 450 bp	(1')
V. fluvialis	942, 353, 180 bp	(1)	370, 218, 180 bp	(2)	503, 450 bp	(2)
V. proteolyticus I	942, 353, 180 bp	(1)	370, 350, 180 bp	(3)	673, 503 bp	(1)
V. proteolyticus II	942, 353, 160, 180 bp	(1)	370, 350, 180 bp		673, 503 bp	
V. vulnificus I	666, 322, 180 bp	(2)	370, 218, 180 bp	(2)	673, 503 bp	(1)
V. vulnificus II	942, 322, 180 bp	(2')	370, 218, 180 bp		673, 503 bp	
V. cholerae	370, 270 bp	(3)	370, 350, 180 bp	(3)	430, 400 bp	(3)
A. hydrophila	600, 390 bp		790, 170 bp		530, 430 bp	
A. salmonicida	600, 390 bp	(4)	790, 170 bp	(4)	530, 430 bp	(4)
Pl. shigelloides	600, 390 bp		790, 170 bp		530, 430 bp	
P. phosphoreum	650, 320 bp	(5)	560, 350 bp	(5)	520, 490 bp	(5)
E. coli	650, 320 bp		410, 370 bp	(6)	520, 490 bp	

[a]Genotypes

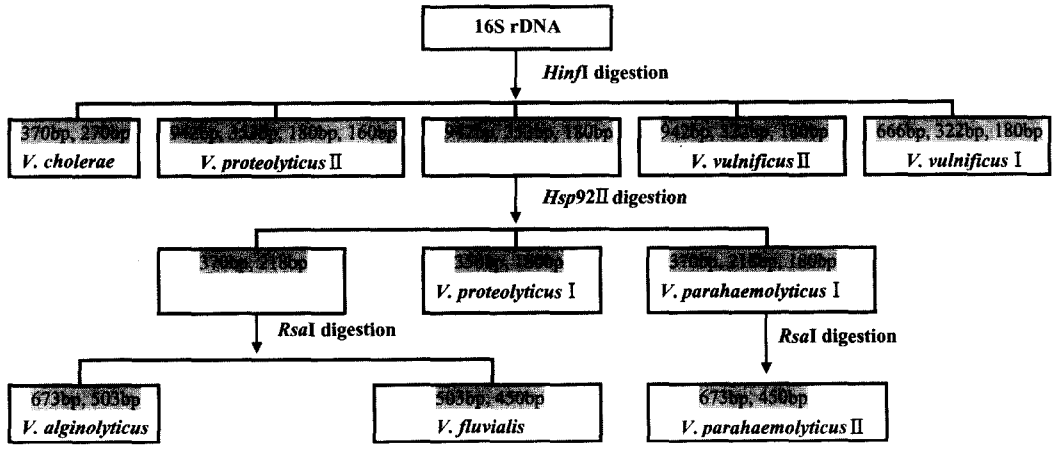

Fig. 5. An identification key for the six *Vibrio* species based on RFLP using three restriction endonucleases.

8 *Vibrio* species, including *V. alginolyticus*, *V. parahaemolyticus*, *V. proteolyticus*, and *V. vulnificus*, were grouped into a single genotype. However, in the present study, each of the *Vibrio* species was assigned to an individual genotype using another set of restriction endonucleases. Predicted RFLPs of other *Vibrio* species (*V. campbellii*, *V. carchariae*, *V. harveyi*, *V. aestuarianus*, *V. diazotrophicus*, *V. anguillarum*, *V. ordalii*, *and V. metchinikovii*) were identical or very similar to those of *V. algiolyticus*, *V. parahaemolyticus*, *V. proteolyticus*, *V. vulni-ficus, and V. fluvialis*, according to the study conducted by Urakawa *et al.* (1999). However, RFLPs of the former group were different from those of the latter 5 species when analyzed by the restriction enzymes used in this study (data not shown). In particular, the latter five species had a 180 bp *Hinf*I fragment, which was not detected in the former group. These two groups of *Vibrio* species were reported to form a large phylogenetic cluster in the genus *Vibrio* based on 16S rDNA sequence (Kita-Tsukamoto *et al.*, 1993). *V. cholerae* and *V. mimicus* were reported to be very closely related to each other, both phenotypically and genotypically (Davis *et al.*, 1981), forming a distinct group in the genus *Vibrio* (Kita-Tsukamoto *et al.*, 1993). However, predicted RFLP of *V. mimicus* ATCC33653 indicated that it had RFLP different from that of *V. cholerae* when digested with *Hinf*I.

Therefore, the nine genotypes of six *Vibrio* species might be identified easily and accurately by following the key established based on their RFLPs of 16S rDNA (Fig. 5). The results also suggest that 16S rDNA RFLP, analyzed using a small number of tetrameric restriction endonucleases, would be sufficient for identification of bacterial strains at the species level, although closer investigation should be carried out for those *Vibrio* species emerging recently. A set of restriction enzymes to be used could be determined by a computer-simulated RFLP analysis as described by Moyer *et al.* (1996).

Acknowledgments

This study was supported by grants from the Korean Ministry of Health and Welfare (HMP-96-F-1-0003) and the University of Incheon in the year of 2000.

References

Alsina, M. and A.R. Blanch. 1994. A set of keys for biochemical identification of environmental *Vibrio* species. *J. Appl. Bacteriol.* 76, 70-85.

Austin, B. and J.V. Lee. 1992. Aeromonadaceae and Vibrionaceae. *In* R.G. Board, D. Jones and F.A. Skinner (eds.). Identification Methods in Applied and Environmental Microbiology. Society for Applied Bacteriology Technical Series No. 29. Blackwell Scientific Publication, Oxford.

Barry, T., R. Powell, and F. Gannon. 1990. A general method to generate DNA probes for microorganisms. *Biotechnology.* 8, 233-236.

Bryant, T.N., J.V. Lee, P.A. West, and R.R. Colwell. 1986. Numerical classification of species of *Vibrio* and related genera. *J. Appl. Bacteriol.* 61, 437-467.

Choudhury, S.R., R.K. Bhadra, and J. Das. 1994. Genome size and restriction fragment length polymorphism analysis of *Vibrio cholerae* strains belonging to different serovar and biotypes. *FEMS Microbiol. Lett.* 115, 329-334.

Davis, B.R., G.R. Fanning, J.M. Madden, A.G. Steigerwalt, H.B. Bradford, Jr., H.L. Smithe, Jr., and D.J. Brenner. 1981. Characterization of biochemically atypical *Vibrio cholerae* strains and designation of a new pathogenic species, *Vibrio mimicus*. *J. Clin. Microbiol.* 14, 631-639.

Dorsch, M., D. Lane, and E. Stackebrandt. 1992. Towards a phylogeny of the genus *Vibrio* based on 16S rRNA sequences. *Int. J. Syst. Bacteriol.* 42, 58-63.

Graham, P.H., M.J. Sadowsky, H.H. Keiser, Y.M. Barnet, R.S. Bradley, J.E. Cooper, D.J. De Ley, B.D. W. Jarvis, E.B. Roslycky, B.W. Strijdom, and J.P.W. Young. 1991. Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. *Int. J. Syst. Bacteriol.* 41, 582-587.

Holt, J.G., N.R. Krieg, P.H.A. Sneath, J.T. Staley, and S.T. Williams. 1994. Facultatively anaerobic gram-negative rods. p. 175-289. *In* R.E. Buchanan and N.E. Gibbons (eds.), Bergey's Manual of Determinative Bacteriology 9th ed. Williams and Wilkins, Baltimore, Maryland.

Kita-Tsukamoto, K., H. Oyaizu, K. Nanba, and U. Simidu. 1993. Phylogenetic relationships of marine bacteria, mainly members of the family Vibrionaceae, determined on the basis of 16S rRNA sequences. *Int. J. Syst. Bacteriol.* 43, 8-19.

Lotz, M.J., M.L. Tamplin, and G.E. Rodrick. 1983. Thiosulfate-citrate-bile salts-sucrose agar and its selectivity for clinical and marine *Vibrio* organisms. *Ann. Clin. Lab. Sci.* 13, 45-48.

Moyer, C.L., F.C. Dobbs, and D.M. Karl. 1994. Phylogenetic diversity of the bacterial community from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Appl. Environ. Microbiol.* 60, 871-879.

Moyer C.L., J.M. Tiedje, F.C. Dobbs, and D.M. Karl. 1996. A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. *Appl. Environ. Microbiol.* 62, 2501-2507.

Navarro, E., P. Simonet, P. Normand, and R. Bardin. 1992. Characterization of natural populations of *Nitrobacter* species using PCR/RFLP analysis of the ribosomal intergenic spacer. *Arch. Microbiol.* 157, 107-115.

Östling, J., H. Louise, K. Flardh, B. Svenblad, A. Jouper-Jaan, and S. Kjelleberg. 1993. Starvation and Recovery of *Vibrio*. p. 103-127. *In* S. Kjelleberg (ed.), Starvation in Bacteria., Plenum Press, New York.

Park, Y., Y. Choi, and B. Min. 2003. PCR-DGGE and PCR-RFLP analyses of the internal transcribed space DNA in the Genus *Rhizopus*. *J. Microbiol.* 41, 157-160.

Ralph, D., M. MaClelland, J. Welsh, G. Baranton, and P. Perolat. 1993. *Leptospira* species categorized by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphisms in PCR-amplified rRNA genes. *J. Bacteriol.* 175, 973-981.

Sambrook, J., E.F. Fritsch, and T. Mamiatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *J. Mol. Evol.* 4, 406-425.

Simidu, U., E. Kaneko, and N. Taga. 1977. Microbiological studies of Tokyo Bay. *Microbiol. Ecol.* 3, 173-191.

Shirai, H., M. Nishibuchi, T. Ramamurthy, S.K. Bhattacharya, S.C. Pal, and Y. Takeda. 1991. Polymerase chain reaction for detection of the cholera enterotoxin operon of *V. cholerae*. *J. Clin. Microbiol.* 29, 2517-2521.

Simidu, U., N. Taga, R.R. Colwell, and J.R. Schwartz. 1980. Heterotrophic bacterial flora of the seawater from the Nansei Shoto (Ryukyu Retto) area. *Bull. Jpn. Soc. Sci. Fish.* 46, 505-510.

Urakawa, H., K. Kita-Tsukamoto, and K. Ohwada. 1999. 16S rDNA restriction fragment length polymorphism analysis of psychrotrophic *Vibrios* from Japanese coastal water. *Can. J. Microbiol.* 45(12), 1001-1007.

Warner, J.M. and J.D. Oliver. 1999. Randomly amplified polymorphic DNA analysis of clinical and environmental isolates of *Vibrio vulnificus* and other *Vibrio* species. *Appl. Environ. Microbiol.* 65(3), 1141-1144.

Weisburg, W.G., S.M. Barns, D.A. Pelletier, and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697-703.

West, P.A., P.R. Brayton, T.N. Bryant, and R.R. Colwell. 1986. Numerical taxonomy of *Vibrios* isolated from aquatic environments. *Int. J. Syst. Bacteriol.* 36, 531-541.

Yoon, Y.J., J.W. Kim, Y.K. Koh, and Y.H. Koh. 1996. The diversity of *Vibrio* species collected from coastal sea-water. *Microorg. Ind.* 22, 193-204.