J. Microbiol. Biotechnol. (1999), **9**(1), 78–83

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

The Korean Society for Applied Microbiology

Intraspecific Variation of Environmental and Clinical *Vibrio vulnificus* Isolates as Demonstrated by Restriction Endonuclease Digestion Profiles

KIM, KI YONG[1], HO CHUL YANG[1], MARK L. TAMPLIN[2], AND SANG HO CHOI[1]*

[1]*Department of Food Science and Technology, Institute of Biotechnology and Center for Specialized Agricultural Sciences, Chonnam National University, Kwang-Ju 500-757, Korea*

[2]*Department of Food Science and Human Nutrition, Department of Microbiology and Cell Science, Institute of Food and Agricultural Science, University of Florida, Gainesville, FL32611, U.S.A.*

Received: September 17, 1998

Abstract Thirty-six environmental isolates of *Vibrio vulnificus* obtained from seawater, sediments, and raw seafoods, and 18 clinical isolates from *Vibrio* septicemia patients were typed by restriction endonuclease digestion profiles (REDP) of genomic DNA with *Sfi*I. The results revealed a high-level of variation in REDPs, indicating a vast genomic diversity among *V. vulnificus* strains. Genetic relatedness of the strains showed similarities ranging from 10% to 100%. Different REDPs for isolates from various raw seafoods were obtained, and clustering of strains according to type of seafoods was not observed. In contrast, clinical isolates of *V. vulnificus* showed higher similarity to one another, and could be subdivided into one separate group. The difference in REDPs of the *V. vulnificus* isolates from clinical origin and from raw seafoods substantiates the previous observation that only a single type of pathogenic strain was involved in each human infection, despite the numerous genetically polymorphic strains found from implicated oysters.

Key words: *Vibrio vulnificus*, REDP, genomic polymorphism

The pathogenic marine bacterium *Vibrio vulnificus* can be isolated from seawater and the estuarine environment [16, 22]. It also occurs in raw seafoods including oysters and fish from coastal waters [15, 23]. *V. vulnificus* has been identified as the causative agent of food-borne diseases such as gastroenteritis and life-threatening septicemia in immuno-compromised individuals [3, 10]. It also has been reported that wound infections have resulted from exposure to seawater or from handling of shellfish contaminated with the organism. Mortality from septicemia is quite high (exceeding 50%), and death may occur as fast as within 1 to 2 days after the first signs of illness [15, 19].

*Corresponding author
Phone: 82-62-530-2146; Fax: 82-62-530-2149;
E-mail: shchoi@chonnam.chonnam.ac.kr

As found in many other pathogenic bacteria, the species *V. vulnificus* is comprised of a diverse array of strains. The strains are remarkably variable in many phenotypic and genetic traits, and even show wide variations in virulence. Therefore, proper differentiation and characterization of *V. vulnificus* at the subspecies level has been demanded for epidemiological purposes. Since the first attempt to distinguish *V. vulnificus* into two biotypes based on the host range [21], biochemical and/or serological methods that define several phenotypic features were widely used for intraspecific differentiation of the pathogen [2, 14]. Although these early studies provided valuable methods to distinguish variable phenotypic features, the methods are less discriminatory and used for primary identification or broad differentiation of the pathogen [2, 14].

Recently, methods have been developed for comparison of strains by looking directly at their genomic DNA rather than at phenotypes. Among them, DNA fingerprinting technologies such as REDP (restriction endonuclease digestion profiles), RAPD (random amplified polymorphic DNA), and ribotyping are currently used for reliable differentiation of *V. vulnificus* strains [1, 4, 6, 20]. REDP-PFGE (pulse field gel electrophoresis) analysis differentiates *V. vulnificus* cells found in individual oysters into numerous genetically polymorphic strains [4]. Additionally, this technique gives opportunities to the epidemiologist to trace specific strains from human vibriosis back to the implicated seafoods. It has been demonstrated that a single strain of *V. vulnificus* is predominantly identified in the blood of an infected individual despite the multiple strains found in the implicated oyster (*Crassostrea virginica*) consumed [20]. It has been suggested that the disease was caused by one or a few virulent *V. vulnificus* strains among hundreds of strains present in oysters. Therefore, the infectious dose must be determined for the specific strains, and not the total *V. vulnificus* number in oysters [8].

Compared with the substantial number of reports on genetic variability of *V. vulnificus* strains in contaminated oysters, very little is known about the genetic diversity of the pathogen from other seafoods. Besides oysters, other seafoods such as small octopi (*Octopus variabilis*), arkshells (*Anadara broughtonii*), rockfish (*Sebastes inermis*), conger eels (*Anago anago*), thin-shelled surf clams (*Ruditapes philippinarum*), blue crabs (*Portunus trituberculatus*), and shrimp (*Penaeus japonicus*) are commonly consumed raw or undercooked in Korea and Japan [7, 9]. As a result, increasing numbers of outbreaks of fatal septicemia due to consumption of these seafoods have been reported in Korea (Joon Haeng Rhee, Personal communication, Chonnam University Hospital, Kwang-ju, Korea). In this study, extensive analysis of the genetic variability of *V. vulnificus* isolates from these seafoods, associated seawater, and sediments collected on the southwestern coast of Korea was carried out using REDP-PFGE. The REDPs of these *V. vulnificus* strains were also compared to isolates from human *Vibrio* septicemia cases in Korea. Examination of the genetic diversity of *V. vulnificus* strains and their persistence in particular seafoods is essential to understand whether individual strains or subsets of strains are associated with particular types of seafoods. Monitoring specific *V. vulnificus* strains from the septicemia patients and comparison with isolates from raw seafoods will help to trace the transmission route of the human pathogenic strains, and to confirm the causative food sources.

MATERIALS AND METHODS

Bacterial Isolates and Culture Conditions

A total of 54 strains of *V. vulnificus* were used in this study and their origins are listed in Table 1. Thirty-six environmental isolates were obtained from seawater, sediment samples, and various raw seafoods collected from the southwestern coast of Korea during the summer of 1996 or 1997 (Fig. 1). Eighteen clinical strains, isolated from patients of *Vibrio* septicemia, were generously provided by Dr. Joon Haeng Rhee (Chonnam University Medical School, Kwang-Ju, Korea). For primary identification, the isolates were characterized by conducting standard physiological and biochemical tests with an API 20E kit (Bio Meriux SA, France) [18], and confirmed as *V. vulnificus* by testing for hemolysin production using ELISA [13]. PCR amplification of the *vvhA* gene [22], specific to *V. vulnificus*, was also used for confirmation of the isolates [11, 12]. Most strains had opaque colony morphotypes on brain heart infusion (BHI) agar, with the exception of a few strains as indicated in Table 1.

After identification, all *V. vulnificus* strains were grown in BHI broth optimized by adding 1% salt, and kept

Table 1. Designations, sources, and relevant characteristics of *V. vulnificus* isolates analyzed by REDP in this study.

Strain	Isolation site	Source	Morphotype	PCR amplification[b]
Environmental isolates				
SC9716	Changhung	seawater	T	+
SC9717	Changhung	sediment	O	+
SC9720	Kangjin	sediment	O	+
SC9721	Kangjin	seawater	O	+
SC9722	Kangjin	sediment	O	+
SC9724	Changhung	sediment	T	+
SC9763	Changhung	seawater	T	+
SC9793	Yongkwang	seawater	O	+
SC9794	Yongkwang	sediment	O	+
SC9795	Yongkwang	seawater	O	+
SC9728	Mokpo	seawater	T	+
SC9729	Mokpo	seawater	O	+
SC9730	Mokpo	sediment	O	+
SC9734	Shinan	seawater	O	+
SC9737	Shinan	sediment	O	+
SC9740	Sunchon	seawater	T	+
SC97114	Posung	sediment	O	+
SC97118	Posung	sediment	O	+
SC9613	Yongkwang	crab	T	+
SC9622	Kangjin	small octopus	O	+
SC9624	Kangjin	conch	O	+
SC9625	Kangjin	blue crab	O	+
SC9629	Changhung	thin-shelled surf clam	T	+
SC9631	Kangjin	thin-shelled surf clam	O	+
SC9641	Hampyong	small octopus	T	+
SC9648	Changhung	clam	O	+
SC9649	Mokpo	turban shell	O	+
SC9725	Changhung	thin-shelled surf clam	O	+
SC9733	Mokpo	rock fish	O	+
SC9738	Shinan	oyster	O	+
SC9761	Wando	oyster	O	+
SC9766	Changhung	thin-shelled surf clam	O	+
SC9771	Mokpo	ark shell	O	+
SC97100	Kohung	conch	O	+
SC97116	Posung	crab	T	+
SC97126	Muan	oyster	O	+
Clinical isolates				
CN7	The Chonnam	Human	T	+
CN8	National Univ.	Human	O	+
CN9	Hospital,	Human	O	+
CNUH943		Human	O	+
CNUH944		Human	O	+
CNUH946		Human	O	+
CS91133		Human	O	+
V15		Human	T	+
V16		Human	O	+
V19		Human	O	+
WK3	Wonkwang	Human	O	+
WK6	Univ.	Human	O	+
WK13	Hospital,	Human	O	+
WK15		Human	O	+
WK16		Human	T	+
WK18		Human	O	+
WK20		Human	O	+
WK22		Human	O	+

[a] O, Opaque; T, Translucent.

[b] Determined from observation of characteristic 704-bp band by gel electrophoresis analysis of PCR products [11].

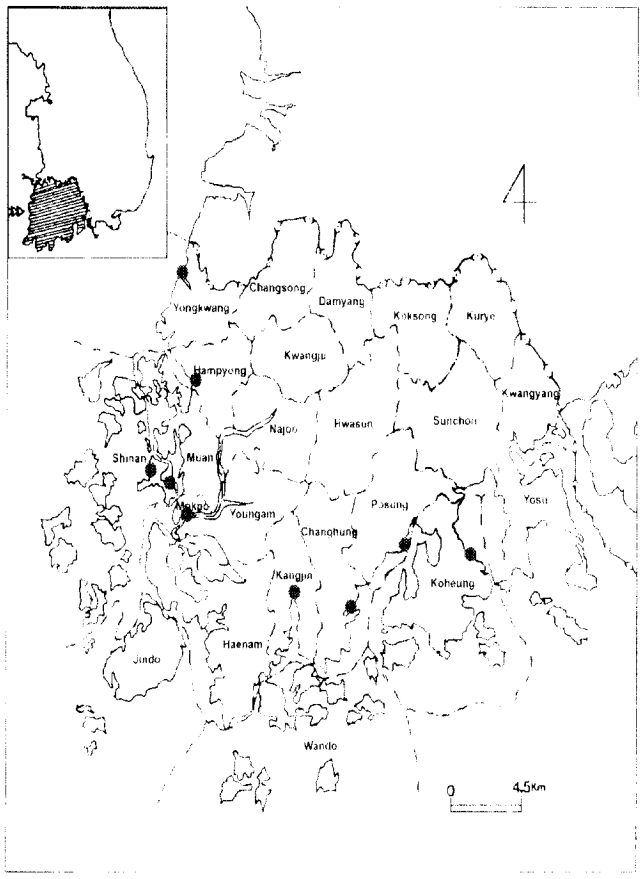

Fig. 1. Map of southwestern coast of Korea showing sampling locations for environmental isolates of *V. vulnificus*.
The circles represent the sites where *V. vulnificus* was often isolated.

frozen at −70°C with 5% dimethylsulfoxide (DMSO) until later use.

Enzyme and Chemicals

The restriction enzyme *Sfi*I and agarose were purchased from New England Biolabs (Beverly, U.S.A.) and Gibco BRL (Gaithersburg, U.S.A.), respectively. They were used as suggested by the suppliers. Reagents for the media were purchased from Difco (Detroit, U.S.A.) and chemicals from Sigma (St. Louis, U.S.A.) at the highest purity available.

Isolation, Digestion, and Fractionation of Genomic DNA

A single colony of each strain was inoculated into 10 ml of BHI broth containing 1% NaCl and incubated for 4~5 h with shaking at 37°C. Then, the cells were harvested, washed twice with phosphate buffer, and resuspended in 0.5 to 1.0 ml of 1 M NaCl-10 mM Tris-50 mM EDTA to adjust to the appropriate density. The resuspended cells were mixed with an equal volume of 1% low melting point agarose for preparation of agarose plugs. The plugs containing cells of *V. vulnificus* were divided to fit into the wells with glass cover slips, and incubated in 1 ml of deproteination solution (2 mg proteinase K with 0.5 M EDTA and 0.5% *N*-laurylsarcosine) at 50°C overnight. Then, 4 ml of PMSF (phenylmethylsulfonylfluoride) solution (40 μg/ml) were added to inactivate the proteinase K and the resulting mixture was incubated for 30 min at 55°C. The plugs were then washed twice with 4 ml of TE buffer (10 mM Tris-Cl, 1 mM EDTA). Genomic DNA within the agarose plugs was digested with the endonuclease *Sfi*I. High molecular weight restriction fragments were resolved using a CHEF (Clamped Homogeneous Electric Field-DRII, Bio-Rad, Richmond, U.S.A.) PFGE system with 1% electrophoresis-grade agarose. Running conditions for PFGE were 200 volts for 24 h at a temperature range between 14~18°C. Switching pulses were used ranging from 5 sec of starting pulse to 35 sec of final pulse. Lambda concatemers were used as DNA molecular size markers. The separated bands in the gel were visualized with a UV transilluminater.

Analysis of REDP Similarity

The size of DNA bands fractionated by PFGE were measured visually and calculated with regression analysis against size standards run concurrently. Then, the presence or absence of bands was translated into binary scores. Similarity indices between pairs of strains were calculated using Dice's coincidence index [5]. The REDP similarities for strains x and y, S_{xy}, equal to the number of bands common in both strains (n_{xy}) divided by the average number of bands in strains X (n_x) and Y (n_y). [$S_{xy} = 2n_{xy}/(n_x+n_y)$]. A clustering analysis was carried out with average-linkage methods to examine the relationship among strains and to infer a dendogram [17]. Data analysis was carried out with a statistical analysis system (SAS Institute, Cary, U.S.A.), and a cluster dendogram and PFGE profiles were plotted by using the S-plus software (Statistical Service, Inc., Seattle, U.S.A.).

Results and Discussion

Forty one *V. vulnificus* isolates originated from different environmental samples such as seafoods, sediments, and seawater, and twenty isolates from human *Vibrio* septicemia cases were typed by REDP-PFGE analysis. We observed that *Sfi*I digestion of DNA from *V. vulnificus* strains generated the appropriate number and best distribution of restriction fragments [4]. Therefore, *Sfi*I was used and the digests yielded up to 23 visible bands ranging in molecular size from 20 to 630.5 kb. The PFGE analysis revealed stable and reproducible REDPs when some strains were repeated. However, 7 of 61 isolates were untypeable

since their DNA persistently produced smears and no REDP could be determined. The degradation of DNA might have been caused by endogenous nucleases or other properties specific to these strains.

The 54 REDPs from the *Sfi*I digests of *V. vulnificus* genomic DNA were divided into 52 distinguished REDP types. The band profiles were converted into binary scores for more precise comparisons and are depicted in Fig. 2. A high-level of diversity among the REDPs of the 36 environmental *V. vulnificus* isolates was observed. All isolates from sediments, seawater, and various seafoods yielded respective REDP types with *Sfi*I, and no REDP was common among these environmental isolates. This is in agreement with the previous report of Tamplin and collaborators who demonstrated numerous genetically polymorphic strains in various environmental samples [4].

Specific grouping of strains was not revealed by visual comparisons of REDPs with *Sfi*I. Therefore, genetic relatedness among strains was examined by calculation of similarity matrices as mentioned in Materials and Methods. The results showed that the similarities ranged from 10% to 100% for all the REDPs analyzed. However, it was apparent that the majority of strains had similarity indices of less than 50% with *Sfi*I, indicating that great genomic diversity exists among *V. vulnificus* strains. In addition, there was no linkage between the REDPs and type of colony morphology (Fig. 3). It is not uncommon to encounter variability in REDP among *V. vulnificus* strains isolated from different types of specimens. For strains isolated from environmental samples, the REDP similarity ranged from 20% to 80%. At any rate, these results also suggest that *V. vulnificus* strains in environmental samples are very heterogeneous. Specific clustering of strains according to type of seafood was not observed, indicating that some individual strains or group of strains are not more frequently associated with a particular type of raw seafoods. *V. vulnificus* strains from various geographical locations also displayed differences in REDPs with *Sfi*I.

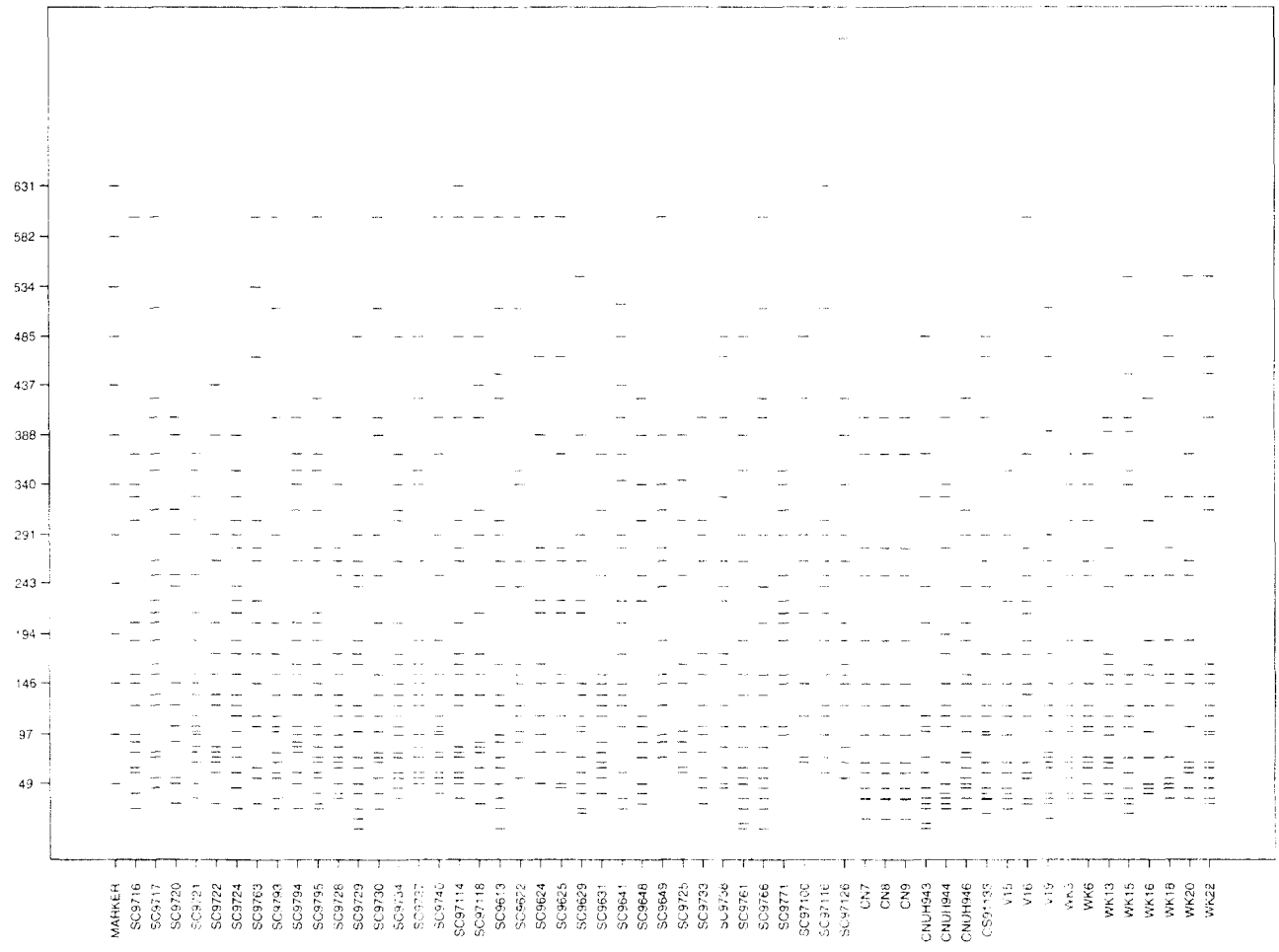

Fig. 2. Diagram of *Sfi*I REPDs of 54 *V. vulnificus* strains. PFGE band patterns of *Sfi*I digests of genomic DNA from each strain were recorded in binary scores.

The migrations of λ DNA concatemers are indicated to the left in kb as molecular size standards.

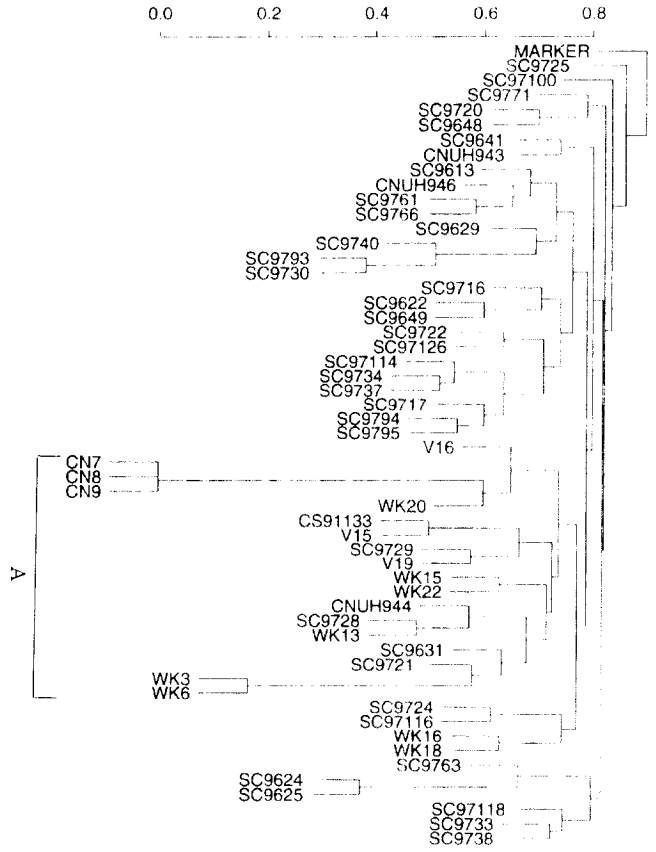

Fig. 3. Dendogram based on *Sfi*I REPDs cluster analysis of 54 *V. vulnificus* strains.

Numbers on the left axis indicate dissimilarity (0.0–100 similarity) determined by using Dice's coincidence index.

Grouping of the REDPs with similarities higher than 30% did not separate the isolates into distinct environmental and clinical isolates. However, a higher degree of homology was observed for clinical than for environmental isolates. Analysis of REDPs of groups with approximately 25% similarity showed that 78% of all *V. vulnificus* populations of clinical origin (14 among 18 strains) can be subdivided into one separate group (cluster A). Among the strains in cluster A, CN7, CN8, and CN9 had identical REDPs with *Sfi*I. This homology was expected because both CN7 and CN9 strains are spontaneous mutants of CN8 with variations in colonial morphology. Apparently, CN7 is more translucent and CN9 is more opaque in colony morphotype than CN8, but they very likely differ in other physiological characteristics and possibly show different REDPs if other endonucleases are used. In addition, strains WK3 and WK9 appeared to be similar at approximately 88%. These strains were isolated from two separate clinical cases of septicemic vibriosis in Chonbuk Province, Korea. The higher similarity observed among clinical isolates is in agreement with the previous observation that only a few type of strains were associated with human *Vibrio* septicemia cases among hundreds of heterogeneous *V. vulnificus* strains found from the implicated oysters [8]. The grouping observed in clinical isolates was not found in the isolates of environmental origin of which only three belonged to cluster A.

Apparently, REDPs between the *V. vulnificus* populations from humans and those of isolates from raw seafoods are different. Although other explanations are possible, this difference indicates that the *V. vulnificus* strains present in raw seafood is rarely involved in human infections. Most environmental isolates differ substantially from isolates from patients in characteristics associated with their pathogenicity. This difference in virulence may also explain why REDPs of isolates from raw seafoods do not match those of clinical isolates despite the frequent contamination and growing consumption of raw seafoods in Korea. This result emphasizes the need to take into account the number of specific pathogenic strains, not the total *V. vulnificus* numbers, when the infective dose is determined. Additionally, opportunities for public health agencies to differentiate *V. vulnificus* strains according to their pathogenicity, and to trace the pathogenic strains from human infections back to the implicated seafoods, will ensure the appropriate response to minimize the potential health hazards presented by the pathogenic strains.

Acknowledgments

We are indebted to Dr. Joon Haeng Rhee for providing the clinical isolates of *V. vulnificus*. This study was supported by a grant to S. H. Choi from the KOSEF (96-0402-01-3), Republic of Korea.

References

1. Arias, R. C., L. Verdonck, J. Swings, E. Garay, and R. Aznar. 1997. Intraspecific differentiation of *Vibrio vulnificus* biotypes by amplified fragment length polymorphism and ribotyping. *Appl. Environ. Microbiol.* **63:** 2600–2606.
2. Biosca, E. G., C. Aramo, J. L. Larsen, and K. Pedersen. 1997. Phenotypic and genotypic characterization of *Vibrio vulnificus*: Proposal for substitution of the subspecific taxon biotype for serovar. *Appl. Environ. Microbiol.* **63:** 1460–1466.
3. Blake, P. A., R. E. Weaver, and D. G. Hollis. 1980. Diseases of humans (other than cholera) caused by *Vibrios*. *Annu. Rev. Microbiol.* **34:** 341–367.
4. Buchrieser, C., V. V. Gangar, R. L. Murphree, M. L. Tamplin, and C. W. Kaspar. 1995. Multiple *Vibrio vulnificus* strains in oysters as demonstrated by clamped homogeneous electric field gel electrophoresis. *Appl. Environ. Microbiol.* **61:** 1163–1168.

5. Dice, L. R. 1945. Measures of the amount of ecological association between species. *Ecology* **26:** 297–302.
6. Hoi, L., A. Dalsgarrd, J. L. Larsen, J. M. Warner, and J. D. Oliver. 1997. Comparison of ribotyping and randomly amplified polymorphic DNA PCR for characterization of *Vibrio vulnificus*. *Appl. Environ. Microbiol.* **63:** 1674–1678.
7. Ichthyological Society of Japan. 1981. *Dictionary of Japanese Fish Names and Their Foreign Equivalents*. pp. 884. Sanseido Co., Ltd., Tokyo, Japan.
8. Jackson, J. K., R. L. Murphree, and M. L. Tamplin. 1997. Evidence that mortality from *Vibrio vulnificus* infection results from single strains among heterogeneous populations in shellfish. *J. Clin. Microbiol.* **35:** 2098–2101.
9. Je, J. G. 1989. Korean names of molluses in Korea. *Korean J. Molacol. Suppl.* **1:** 1–91.
10. Klontz, K. C., S. Lieb, M. Schreiber, H. T. Janowski, S. M. Baldy, and R. A. Gunn. 1988. Syndromes of *Vibrio vulnificus* infections: Clinical and epidemiologic features in Florida cases, 1981~1987. *Ann. Intern. Med.* **109:** 318–323.
11. Lee, J. Y. and S. H. Choi. 1995. Rapid and direct detection of *Vibrio vulnificus* in small octopus (*Octopus variabilis*) using polymerase chain reaction. *J. Microbiol. Biotechnol.* **5:** 181–187.
12. Lee, J. Y., J. B. Eun, and S. H. Choi. 1997. Improving detection of *Vibrio vulnificus* in *Octopus variabilis* by PCR. *J. Food Science* **62:** 179–182.
13. Lee, S. E. 1996. Studies on the hemolysin of *Vibrio vulnificus*. Ph. D. thesis. Chonnam National University, Kwang-Ju, Korea.
14. Martin, S. J. and R. J. Siebeling. 1991. Identification of *Vibrio vulnificus* O serovars with antilipopolysaccharide monoclonal antibody. *J. Clin. Microbiol.* **29:** 1684–1688.
15. Oliver, J. D. and J. B. Kaper. 1997. *Vibrio* species, pp. 228–264. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (eds.), *Food Microbiology Fundamentals and Frontiers*. ASM Press, Washington, D.C., U.S.A.
16. Oliver, J. D., R. A. Warner, and D. R. Cleland. 1983. Distribution of *Vibrio vulnificus* and other lactose-fermenting *Vibrios* in the marine environment. *Appl. Environ. Microbiol.* **45:** 985–998.
17. Romesburg, H. S. 1984. *Cluster Analysis for Researchers*. Lifetime Learning Publications, Belmont, CA, U.S.A.
18. Smibert, R. M. and N. R. Krieg. 1994. Phenotypic characterization, pp. 607–654. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (eds.), *Methods for General and Molecular Bacteriology*. American Society for Microbiology, Washington, D.C., U.S.A.
19. Tacket, C. O., F. Brenner, and P. A. Blake. 1984. Clinical features and an epidemiological study of *Vibrio vulnificus* infections. *J. Infect. Dis.* **149:** 558–561.
20. Tamplin, M. L., J. K. Jackson, C. Buchrieser, R. L. Murphree, K. M. Portier, V. Gangar, L. G. Miller, and C. W. Kaspar. 1996. Pulsed-field gel electrophoresis and ribotype profiles of clinical and environmental *Vibrio vulnificus* isolates. *Appl. Environ. Microbiol.* **62:** 3572–3580.
21. Tison, D. L., M. Nishibuchi, J. D. Greenwood, and R. J. Seidler. 1982. *Vibrio vulnificus* biogroup 2: New biogroup pathogenic for eels. *Appl. Environ. Microbiol.* **44:** 640–646.
22. Yamamoto, K., A. C. Wright, J. B. Kaper, and J. G. Morris, Jr. 1990. The cytolysin gene of *Vibrio vulnificus*: Sequence and relationship to the *Vibrio cholerae* El Tor hemolysin gene. *Infect. Immun.* **58:** 2706–2709.
23. Wright, A. C., R. T. Hill, J. A. Johnson, M. C. Roghman, R. R. Colwell, and J. G. Morris, Jr. 1996. Distribution of *Vibrio vulnificus* in the Chesapeake Bay. *Appl. Environ. Microbiol.* **62:** 717–724.