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Direct Identification of *Vibrio vulnificus* by PCR Targeting Elastase Gene

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Abstract A PCR assay for the rapid detection of *Vibrio vulnificus* strains was developed using a virulence gene for elastase found in various *Vibrio* species. The DNA sequences in the elastase gene facilitated the identification of a species-specific probe for pathogenic *V. vulnificus* strains from both clinical and environmental sources. Using an elastase gene-based PCR reaction, a species-specific 507-bp PCR product was visualized by agarose gel electrophoresis. Three different DNA extraction methods were then compared to improve the simplicity and rapidity of detection. A PCR assay using the conventional DNA extraction or boiling method was able to detect as few as 25 *V. vulnificus* cells, making the detection limits at least 1-log-scale lower than that for the EDTA-treated DNA extraction method. In particular, the boiling method, which does not require purification of the chromosomal DNA, was very effective in terms of simple and rapid detection. Meanwhile, the detection limit in a mixed bacterial culture that included other bacteria, such as *Escherichia coli* or *Bacillus subtilis*, was two *V. vulnificus* cells, which was 1-log-scale lower than that for the control. Accordingly, when coupled with a new DNA extraction method, the elastase gene-based PCR can provide a rapid, specific, and sensitive method for identifying *V. vulnificus* in clinical and environmental samples.

Key words: Detection, *Vibrio vulnificus*, PCR, elastase

Vibrio vulnificus, a halophilic marine vibrio, is an opportunistic human pathogen that can cause fatal primary septicemia and severe wound infections. The primary septicemia caused by *V. vulnificus* results in a high mortality up to 60% within one day [12, 21]. Primary septicemia is usually associated with the consumption of raw seafood, and high densities of *Vibrio vulnificus* have been found in the intestinal contents of certain bottom-feeding fish, particularly those that consume mollusks and crustaceans [7]. Individuals who are immunocompromised or who have elevated serum iron levels, typically as a result of liver disease, are mostly at risk of infection from this organism [12]. The most common symptoms of the primary septicemia form of infection include fever, chills, nausea, and hypotension.

The genus *Vibrio* includes more than 30 species, and 12 of these are human pathogens or have been isolated from human clinical specimen [20]. For a definitive diagnosis, *V. vulnificus* must be differentiated from at least 12 other pathogenic *Vibrio* species. *V. vulnificus* can be identified from blood or other clinical samples by culturing on several media including a blood agar and other nonselective agars. The use of differential media, such as a thiosulfate-citrate-bile salts-sucrose (TCBS) agar, has also been recommended when patients have a compatible diarrheal illness and history of eating raw seafood [20]. However, the low recovery rates of clinical *V. vulnificus* strains on a TCBS agar suggest that this medium should not be used for the direct plating of clinical specimens [11]. In addition, since patients infected with primary *V. vulnificus* speticemia usually die within 2 days of being admitted to hospital, a rapid and definitive method for identifying *V. vulnificus* within hours from blood or tissue samples is needed. Currently, serological and molecular methods are used for the rapid identification of this species. For example, an enzyme immunoassay (EIA) in an ELISA format using a *V. vulnificus*-specific monoclonal antibody (mAb) directed against an intracellular epitope of *V. vulnificus* is used and the cell line producing the *V. vulnificus*-specific mAb is available from the American Type Culture Collection. [26, 27].

Molecular techniques, particularly specific oligonucleotide probes, constitute a very sensitive and specific tool for detecting *V. vulnificus*. As such, it has been suggested that primers directed against rRNA genes should be used, since rRNA molecules are essential constituents of all living organisms and present in growing cells in very high numbers [1, 2]. However, the sequences of this region are almost identical among phylogenetically close vibrios. Various researches have already shown that cytolysin, a

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virulence factor, is produced by all *V. vulnificus* strains and is species-specific [15, 24]. Therefore, the sequence of the cytolysin gene has been used to construct primers for PCR identification [3, 5, 18]. Elastase, with a broad substrate specificity, including biologically important host molecules, has also been suggested as an important virulence factor in *V. vulnificus* [9, 14, 25]. Plus, the elastase genes from at least three *Vibrio* species, including *V. vulnificus*, have already been cloned and their sequences analyzed [4, 6, 8, 22].

Accordingly, the current study investigated whether the elastase gene sequence could be used to develop a PCR method for the specific identification of *V. vulnificus* strains. Two specific primers based on the *V. vulnificus* elastase gene were designed and an effective DNA extraction method examined. As a result, *V. vulnificus* was effectively detected from both clinical and environmental sources.

MATERIALS AND METHODS

Bacterial Strains and Media

The strains used in this study and their sources are listed in Table 1. The isolates were routinely grown by streaking on thiosulfate-citrate-bile salts-sucrose (TCBS, Difco, Detroit, MI, U.S.A.) and incubated at 37°C. Single colonies were then streaked in tryptic soy agar (TSA, Difco) supplemented with 1% NaCl and grown in tryptic soy broth (TSB, Difco) supplemented with 1% NaCl.

DNA Extraction from Bacterial Samples

Due to the high mortality resulting from primary septicemia caused by *V. vulnificus* within 24 h, identifying the bacterium within hours is crucial. As such, 3 DNA extraction methods were performed and their efficiencies were compared as regards the specific detection of *V. vulnificus*. The first method was based on the routine chromosomal DNA purification protocol. Briefly, 1.5 ml of the culture was microcentrifuged and resuspended in 500 µl of a TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The chromosomal DNA was released by adding 25 µl of 10% sodium dodecyl sulfate and incubating at 65°C for 10 min. Thereafter, the solution was treated with 20 µg/ml RNaseA at 37°C for 1 h. The DNA was then extracted with the same volume of phenol and the phenol-treated solution extracted by treatment with 25:24:1 phenol-chloroform-isoamyl alcohol. Next, the extracted DNA was precipitated with 16 µl of 5 M NaCl and 2 volumes of absolute ethanol, washed with 70% ethanol, and dried thoroughly. The purity was determined by calculating the A_{260}/A_{280} ratios, while the DNA concentrations were obtained from the A_{260} values (GeneQuant Pro RNA/DNA Calculator, Pharmacia Biotech., Uppsala, Sweden). For the boiling method, the cells were grown in TSB containing 1.5% NaCl at 37°C with shaking at 200 rpm

Table 1. Bacterial strains used to evaluate sensitivity and specificity of elastase-based PCR probe.

Species	Strain[a]	Elastase-based probe
Vibrio vulnificus	ATCC 27562	+
V. vulnificus	KTCC 2962	+
V. vulnificus	KTCC 2980	+
V. vulnificus	KTCC 2981	+
V. vulnificus	KTCC 2982	+
V. vulnificus	KTCC 2983	+
V. vulnificus	KTCC 2985	+
V. vulnificus	KTCC 2986	+
V. vulnificus	KTCC 2987	+
V. vulnificus	PNUH1, clinical isolate from PNUH	+
V. vulnificus	PNUH2, clinical isolate from PNUH	+
V. vulnificus	PNUH3, clinical isolate from PNUH	+
V. alginolyticus	Isolate from shellfish	-
V. campbelli	KCTC 2716	-
V. fluvialis	KCTC 2473	-
V. furnissii	Isolate from shellfish	-
V. mimicus	ATCC 33653	-
V. ordalii	KCTC 2424	-
V. harveyi	KCTC 2717	-
V. parahaemolyticus	ATCC 17802	-
V. parahaemolyticus	Sero type 04, via Japan Disease Center	-
V. parahaemolyticus	Environmental isolate	-
V. parahaemolyticus	Environmental isolate	-
Esherichia coli		-
Bacillus subtilis		-

[a]ATCC, American Type Culture Collection; KCTC, Korean Collection Type Cultures; PNUH, Pusan National University Hospital.

overnight. One milliliter of the culture was boiled for 5 min and centrifuged. The supernatant was then directly used for the PCR reaction. The third method was based on extraction using an EDTA solution. The pellets or colonies were resuspended in 100 µl of a 1 mM EDTA solution. After 5 min of incubation, the solution was centrifuged and the supernatant used for detection. After treatment with one of these extraction methods, 1 µl of the resulting suspension was added to the PCR reaction as the template.

PCR

To develop a PCR method specific for the *V. vulnificus* elastase gene, various oligonucleotide primer sets were tested using the strains listed in Table 1, which included 12 strains of *V. vulnificus*. The primer sequences were selected from the regions that were not conserved between the *V. vulnificus* elastase gene and the elastase gene sequences from other *Vibrio* spp. The selected primer sequences were sense (E1) 5'-AAA-CTC-AGG-TCT-GAT-ATA-CAG-C, and antisense (E2) 5'-AAG-TTG-CTA-CCT-GGC-GTG-TTG from the nucleotide position 1329 to 1835. The target of the primers was a 507-bp DNA fragment specific to the *V. vulnificus* elastase gene. Amplification of the genomic DNA was performed in a total volume of 20 µl. The reaction mixture consisted of 1 µl of the template, 1 U of Taq DNA polymerase, 250 mM of dNTPs, 1.5 mM of $MgCl_2$, 1 µl of each primer (1 pmol/), and a 1× polymerase buffer. For every reaction, the reaction mixture was pre-denatured at 95°C for 5 min to ensure the complete dissociation of the template DNA. The amplification was performed based on 30 cycles of amplification, consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and an extension at 72°C for 1 min. For the last cycle, the elongation was prolonged to 7 min to ensure proper extension of the bases. The dilution of the boiled culture supernatant, annealing temperature, and number of amplification cycles for the PCR were varied and optimized. To rule out a false positive signal, negative controls with all the constituents of the reaction mixture, except the template DNA, were employed for every experiment.

Detection of PCR Product

Five µl from the total 20 µl of the PCR mixture was electrophoresed on 0.8% agarose gel containing 0.2 mg/ml of ethidium bromide. The gels were then run in a Tris-Borate-EDTA (TBE) buffer at 100 V with 5 µl of a standard 100-bp DNA ladder marker.

Sensitivity of Amplification

A single colony of *V. vulnificus* was utilized to assess the sensitivity of the PCR assay. As such, 5 ml of a TSB culture was inoculated with a single colony, incubated for 24 h at 37°C, and diluted with a TSB solution to obtain dilutions over the range of 10^0 to 10^4. The cell concentration was then obtained by measuring the absorbance at 600 nm. The genomic DNA from *V. vulnificus* was diluted with sterile water to a concentration ranging from 50 ng to 5 fg. The PCR and gel electrophoresis were also carried out under the same conditions as described above.

RESULTS

Specificity and Sensitivity of PCR

All the *V. vulnificus* strains isolated from either clinical specimens or the environment were amplified based on the 507-bp fragment using the set of primers under the PCR conditions. Meanwhile, no non-*V. vulnificus* strains or nonspecific products were amplified. As such, the PCR amplification was only found in the *V. vulnificus* strains and not in other bacteria including other *Vibrio* spp., regardless of the template preparation method. Figure 1A

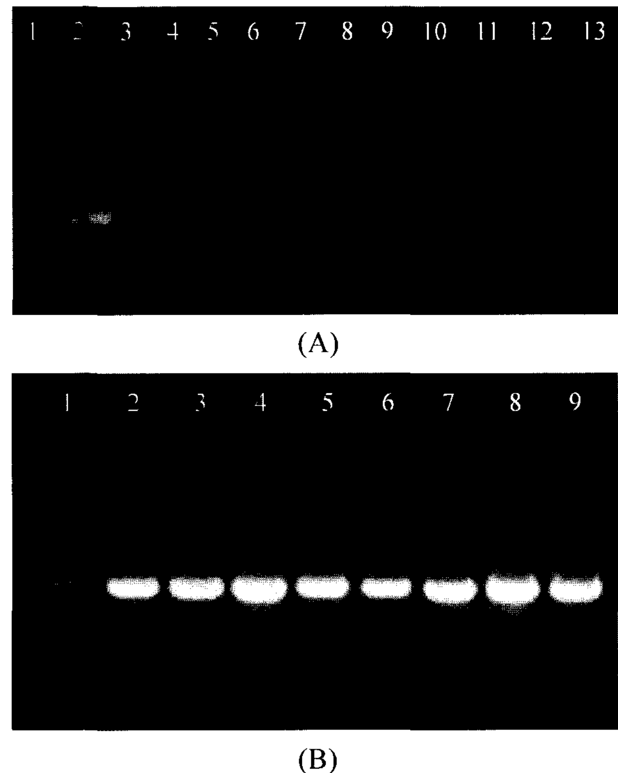


Fig. 1. Identification of *V. vulnificus* by PCR with E1-E2 primer set.

(A) lane 1, 100-bp DNA ladder; lane 2, *V. vulnificus*; lane 3, *V. alginolyticus*; lane 4, *V. campbelli*; lane 5, *V. fluvialis*; lane 6, *V. furnissii*; lane 7, *V. mimicus*; lane 8, *V. ordalii*; lane 9, *V. harveyi*; lane 10; *V. parahaemolyticus*; lane 11, *E. coli*; lane 12, *B. subtilis*; lane 13, negative control (without bacteria). (B) Lane 1, 100-bp DNA ladder; lane 2, *V. vulnificus* KCTC 2962; lane 3, *V. vulnificus* KCTC 2890; lane 4, *V. vulnificus* KCTC 2981; lane 5, *V. vulnificus* KCTC 2986; lane 6, *V. vulnificus* KCTC 2987; lane 7, *V. vulnificus* PNUH1; lane 8, *V. vulnificus* PNUH2; lane 9, *V. vulnificus* PNUH3.

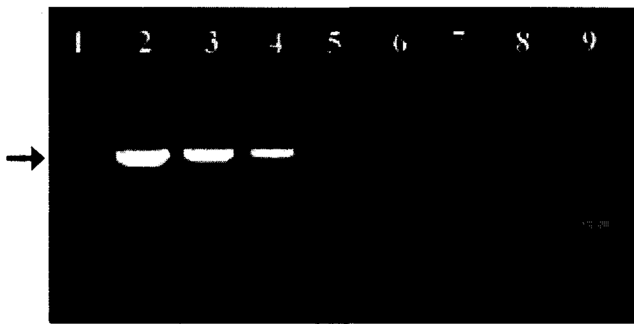


Fig. 2. Sensitivity of PCR with E1-E2 primer set in detection of *V. vulnificus* strains.
The chromosomal DNA was extracted using the conventional DNA extraction method. DNA. Lane 1, 100-bp DNA ladder; lane 2, 50 ng; lane 3, 5 ng; lane 4, 500 pg; lane 5, 50 pg; lane 6, 5 pg; lane 7, 500 fg; lane 8, 50 fg; lane 9, 5 fg.

shows the amplification of the fragment from various *Vibrio* spp. and other bacteria. Only the *V. vulnificus* strains were specifically amplified. All 8 different *V. vulnificus* strains examined exhibited a strong 507-bp DNA fragment band in the agarose gel (Fig. 1B).

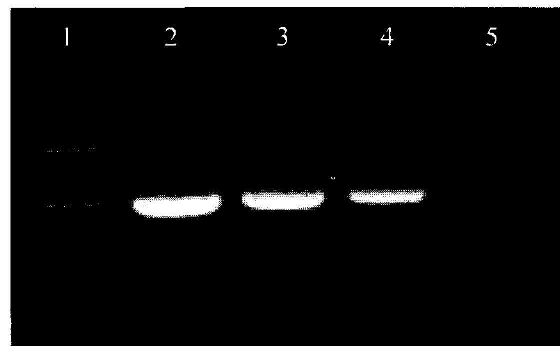
The sensitivity of the primers was tested by performing a PCR with serially diluted *V. vulnificus* chromosomal DNA. Based on the range of dilutions tested, the detection limit of the primer set was 50 pg using ethidium bromide staining (Fig. 2).

Comparison of DNA Extraction Method for Rapid Identification of *V. vulnificus*

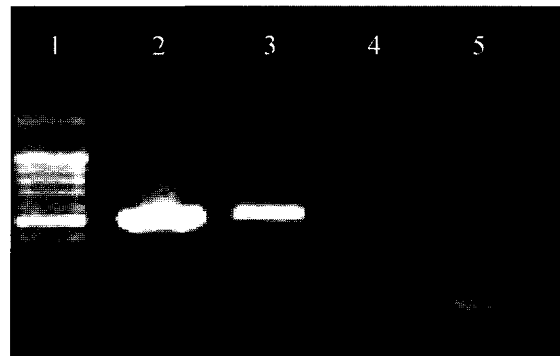
When using the conventional DNA extraction method, as few as 25 *V. vulnificus* cells could be detected by the PCR with these primers (Fig. 3A). The boiling method also resulted in the same sensitivity (Fig 3B). In contrast, the DNA extraction method using a 1 mM EDTA solution was only able to detect about 250-400 cells, i.e. 1-log-scale higher than the conventional DNA extraction and boiling method (Fig. 3C).

Detection of *V. vulnificus* in Mixtures with other Bacteria

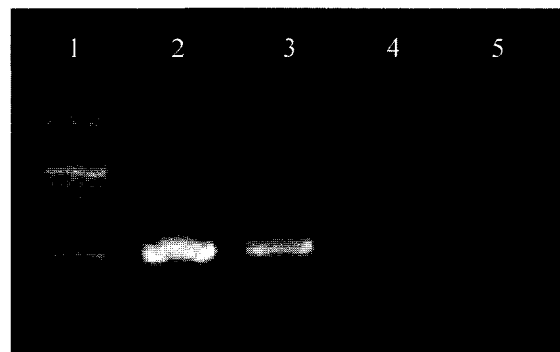
The boiling method described above worked preferentially for *V. vulnificus* when it was mixed with *Escherichia coli* or *Bacillus subtilis* bacteria. Since *E. coli* can possibly contaminate specimens during collection, tenfold serially diluted *V. vulnificus* (4.1×10^8 cells/ml) was mixed with *E. coli* or *B. subtilis* cells (7.2×10^8 cells/ml). As such, 500 of each bacterium was mixed based on a 1:1 (v/v) ratio to make a total volume of 1,000 μl, then a 1 μl aliquot of each dilution sample was added directly to the PCR mixture for amplification. When using the two primers, as few as two *V. vulnificus* cells were detected in the mixed bacterial culture (Fig. 4). making the PCR sensitivity for a mixed bacteria culture 1-log-scale higher than that for the control.



(A)



(B)



(C)

Fig. 3. Sensitivity of PCR in detection of *V. vulnificus* strains using conventional (A), boiling (B), and 1 mM EDTA (c) DNA extraction methods.
Lane 1, 100-bp DNA ladder; lane 2, 10^{-1} dilution (2.5×10^4 cells); lane 3, 10^{-2} (2.5×10^3 cells); lane 4, 10^{-3} (2.5×10^2 cells); lane 5, 10^{-4} (2.5×10^1 cells). The PCR was performed with serial 10-fold dilutions of *V. vulnificus* KCTC 2962 (2.5×10^8 cells/ml) and E1-E2 primers. One microliter of the sample served as the DNA template in the 20-μl PCR mixture.

DISCUSSION

The current study attempted to develop a highly sensitive and specific method for identifying *V. vulnificus* in clinical specimens within several hours. Phenotypic identification methods are influenced by several factors, such as the media components, culture condition, and antibiotics in the

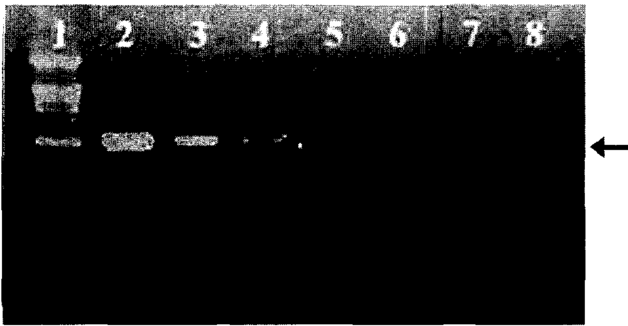


Fig. 4. Identification of *V. vulnificus* by PCR in mixed bacteria culture.

Ten-fold serially diluted *V. vulnificus* (4.1×10^8 cells/ml) was mixed with *E. coli* or *B. subtilis* cells (7.2×10^8 cells/ml). Each bacterium (500 µl) was mixed based on a 1:1 (v/v) ratio to make a total volume of 1,000 µl. One microliter of each dilution sample served as the DNA template in the 20-µl PCR mixture. Lane 1: 100-bp DNA ladder; lane 2 through 8: 10^2, 10^3, 2×10^3, 4×10^3, 8×10^3, 10^4, 10^5 dilutions of *V. vulnificus* KCTC 2962 from 2×10^3 to 2 cells.

specimens. Many Korean septicemia patients suspected of *V. vulnificus* speticemia were negative by bacterial cultures when they were tested for that infection [19]. Therefore, several researchers have recently developed PCR protocols that target the *V. vulnificus*-specific cytotoxin-hemolysin gene to detect the microorganism in various environmental sources [1, 3, 10, 17, 19]. Hill *et al.* used the cytotoxin-hemolysin gene to detect the bacterium in artificially contaminated oysters [10]. However, the sensitivity of this method is low, and overnight incubation is necessary for PCR detection. Meanwhile, Brauns *et al.* developed a method for detecting culturable and nonculturable bacteria in seawater [3], and Arias *et al.* reported on a nested PCR method for the rapid detection of the bacterium in fish, sediments, and water [1], where the latter method can detect as few as 12 to 120 cells in artificially seeded eel homogenates without enrichment. Lee *et al.* also reported on a nested PCR method along with a direct DNA extraction method for rapid detection [19], which were successfully applied to clinical specimens, where approximately 94% of *V. vulnificus* culture-positive specimens tested positive according to the nested PCR.

In contrast, the current study presented a rapid PCR method for detecting *V. vulnificus* based on targeting the elastase gene after examining three different DNA extraction methods. Elastase has been suggested as an important virulence factor in various human pathogenic bacteria [9, 14]. The characteristics of the elastase of *V. vulnificus* as a potential virulence factor have already been studied using purified protein in animal models [16, 23]. The different level of elastase expression depending on the growth phase of *V. vulnificus* has also been examined [13]. Two primers specifically amplifying a 507-bp fragment of *V. vulnificus* were selected. When using these primers internal to the elastase gene, all non-*V. vulnificus* strains responded negatively to the amplification of the elastase gene. In particular, the sensitivity of this method was enhanced 10-fold when *V. vulnificus* was mixed with other bacteria. This high sensitivity in a mixed bacteria culture is still not understood; however, a possible explanation is that the loss of *V. vulnificus* genomic DNA during DNA extraction is decreased, as the overall yield of DNA is increased due to the high concentration of other bacteria. Furthermore, a new boiling method with a shorter diagnosis time produced the same sensitivity as the conventional DNA extraction method, making it a promising candidate for the rapid identification of suspected *V. vulnificus* strains isolated from clinical specimens and food samples implicated as sources of infection for patients with cases of food poisoning. However, the direct application of this method to clinical specimens should initially be seen as a complementary tool to conventional culture methods.

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REFERENCES

1. Arias, C. R., E. Gary, and R. Aznar. 1995. Nested PCR method for rapid and sensitive detection of *Vibrio vulnificus* in fish, sediments, and water. *Appl. Environ. Microbiol.* **61:** 3476-3478.
2. Aznar, R., W. Ludwig, R. I. Amann, and K. H. Schleifer. 1994. Sequence determination of rRNA genes of pathogenic *Vibrio* species and whole-cell identification of *V. vulnificus* with rRNA-targeted oligonucleotide probes. *Int. J. Syst. Bacteriol.* **44:** 330-337.
3. Brauns, L. A., M. C. Hudson, and J. D. Oliver. 1991. Use of the polymerase chain reaction in detection of culturable and nonculturable *Vibrio vulnificus* cells. *Appl. Environ. Microbiol.* **57:** 2651-2655.
4. Cheng, J. C., C. P. Shao, and L. I. Hor. 1996. Cloning and nucleotide sequencing of the protease gene of *Vibrio vulnificus*. *Gene* **183:** 255-257.
5. Coleman, S. S., D. M. Melanson, E. G. Biosca, and J. D. Oliver. 1996. Detection of *V. vulnificus* biotypes 1 and 2 in eels and oysters by PCR amplification. *Appl. Environ. Microbiol.* **62:** 1378-1382.
6. David, V. A., A. H. Deutch, A. Sloma, D. Pawlyk, A. Ally, and D. R. Durham. 1992. Cloning, sequencing and expression

of the gene encoding the extracellular neutral protease, vibriolysin, of *Vibrio proteolyticus*. *Gene* **112:** 107-112.

7. Depaola, A., G. M. Capers, and D. Alexander. 1994. Densities of *Vibrio vulnificus* in the intestines of fish from the U. S. Gulf Coast. *Appl. Environ. Microbiol.* **60:** 984-988.
8. Hase, C. C. and R. A. Finkelstein. 1991. Cloning and nucleotide sequence of the *Vibrio cholerae* hemagglutinin/ protease (HA/protease) gene and construction of an HA/ protease-negative strain. *J. Bacteriol.* **173:** 3311-3317.
9. Hase, C. C. and R. A. Finkelstein. 1993. Bacterial extracellular zinc-containing metalloproteases. *Microbiol. Rev.* **57:** 823-837
10. Hill, W. E., S. P. Keasler, M. W. Trucksess, P. Feng, C. A. Kaysner, and K. A. Lampel. 1991. Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. *Appl. Environ. Microbiol.* **57:** 707-711.
11. Høi, L., I. Dalsgarrd, and A. Dalsgarrd. 1998. Improved isolation of *Vibrio vulnificus* from seawater and sediment with cellobiose-colistin agar. *Appl. Environ. Microbiol.* **64:** 1721-1724.
12. Hlady, W. G. and K, C. Klontz. 1996. The epidemiology of *Vibrio* infections in Florida, 1981-1993. *J. Infect. Dis.* **173:** 1176-1183.
13. Jeong. H. S., K. H. Jeong, H. K. Choi, K. J. Park, K. H. Lee, J. H. Rhee, and S. H. Choi. 2001. Differential expression of *Vibrio vulnificus* elastase gene in a growth phase-dependent manner by two different types of promoters. *J. Biol. Chem.* **276:** 13875-13880.
14. Jeong, K. C., H. S. Jeong, J. H. Rhee, S. E. Lee, S. S. Chung, A. M. Starks, G. M. Escudero, P. A. Gulig, and S. H. Choi. 2000. Construction and phenotype evaluation of a *Vibrio vulnificus vvpE* mutant for elastolytic protease. *Infect. Immun.* **68:** 5096-5106.
15. Kaysner, C. A., C. J. Abeyta, M. M. Wekell, A. DePaola, R. F. Stott, and J. M. Leitch. 1987. Virulent strains of *Vibrio vulnificus* isolated from estuaries of the United States West Coast. *Appl. Environ. Microbiol.* **53:** 1349-1351.
16. Kothary, M. H. and A. S. Kreger. 1987. Purification and characterization of an elastolytic protease of *Vibrio vulnificus*. *J. Gen. Microbiol.* **133:** 1783-1791.
17. Lee, J. H., K.-H. Lee, and S. H. Choi. 2001. Enumeration of *Vibrio vulnificus* in natural samples by colony blot hybridization. *J. Microbiol. Biotechnol.* **11:** 302-309.
18. Lee. S. E., S. Y. Kim, S. J. Kim, H. S. Kim, J. H. Shin, S. H. Choi, S. S. Chung, and J. H. Rhee. 1998. Direct identification of *Vibrio vulnificus* in clinical specimens by nested PCR. *J. Clin. Microbiol.* **36:** 2887-2892.
19. Lee, S. H. and S. J. Kim. 1989. Epidemiologic and microbiologic study in 64 clinically suspected cases of *V. vulnificus* infections. *Kor. J. Int. Med.* **36:** 820-830.
20. McLaughlin, J. C. 1995. *Vibrio*, pp. 465-475. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (eds.), *Manual of clinical microbiology*, 6th ed. American Society for Microbiology, Washington, D.C., U.S.A.
21. Oliver, J. D. 1989. *Vibrio vulnificus*, pp. 569-600. *In* M. P. Doyle, (ed.), *Foodborne Bacterial Pathogens*, Marcel Dekker, Inc., New York, U.S.A.
22. Milton, D. L., A. Norqvist, and H. Wolf-Watz. 1992. Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum*. *J. Bacteriol.* **174:** 7235-7244.
23. Miyoshi, S. and S. Shinoda. 1988. Role of the protease in the permeability enhancement by *Vibrio vulnificus*. *Microbiol. Immunol.* **32:** 1025-1032.
24. Morris, J. G., A. C. Wright, D. M. Roberts, P. K. Wood, L. M. Simpson, and J. D. Oliver. 1987. Identification of environmental *Vibrio vulnificus* isolates with a DNA probe for the cytotoxin-hemolysin gene. *Appl. Environ. Microbiol.* **53:** 193-195.
25. Rhee, J. E., J. H. Lee, H. S. Jeong, U. Park, D.-H. Lee, G.-J. Woo, S.-I. Miyoshi, and S. H. Choi. 2003. Evidence that temporally alternative expression of the *Vibrio vulnificus* elastase prevents proteolytic inactivation of hemolysin. *J. Microbiol. Biotechnol.* **13:** 1021-1026.
26. Tamplin. M. L., A. L. Martin, A. D. Ruple, D. W. Cook, and C. W. Kasper. 1991. Enzyme immunoassay for identification of *Vibrio vulnificus* in seawater, sediment, and oysters. *Appl. Environ. Microbiol.* **57:** 1235-1240.
27. U. S. Food and Drug Administration. 1995. *Bacteriological and Analytical Manual*. Association of Official Analytical Chemists, Arlington, VA.