CURRENT MICROBIOLOGY Vol. 49 (2004), pp. 175–179
DOI: 10.1007/s00284-004-4288-5

Current Microbiology
An International Journal

Characterization and Virulence of Hemolysin III from *Vibrio vulnificus*

Yu-Chung Chen,[1] Ming-Chung Chang,[2] Yin-Ching Chuang,[3] Chii-Ling Jeang[1]

[1]Department of Food Science, National Chung-Hsing University, 250 Kuokuang Road, Taichung 402, Taiwan
[2]Department of Biochemistry, National Cheng-Kung University, Medical College, Tainan 701, Taiwan
[3]Department of Medicine and Medical Research, Chi-Mei Foundation Medical Center, No. 901, Chung Hwa Road, Yung Kang City 710, Tainan, Taiwan

Received: 9 December 2003 / Accepted: 27 February 2004

Abstract. *Vibrio vulnificus*, a highly virulent marine bacterium, is the causative agent of both serious wound infections and fatal septicemia in many areas of the word. A gene (*hlyIII*) encoding a hemolysin was cloned and sequenced from *V. vulnificus*. Nucleotide sequence analysis predicted an open reading frame of 642 bp encoding a 214 amino acid polypeptide that showed 48% sequence identity to the hemolysin III of *Bacillus cereus*. When HlyIII of *V. vulnificus* was expressed in *Escherichia coli*, crude extracts exhibited hemolytic activity similar to that of hemolysin III from *Bacillus cereus*. A *hlyIII* isogenic mutant was constructed via insertional inactivation and showed an attenuated virulence compared with the wild-type strain when this mutant was administered intraperitoneally in mice.

Vibrio vulnificus is a halophilic Gram-negative bacterium that has emerged as an increasingly important pathogen capable of causing both serious wound infections and fatal septicemia in humans [4, 8, 21, 25]. Primary septicemia may be acquired by consuming seafood containing this organism, with a mortality rate exceeding 50%. Infections are associated with exposure of wounds to seawater, with a mortality rate of about 25% [5, 8, 15]. Although the pathogenic mechanism of *V. vulnificus* infection has not been fully delineated, several potential virulence factors such as capsule polysaccharide (CPS) [26, 28, 32], iron-sequestering systems [18], metalloprotease [16] and type IV leader peptidase-N-methyltransferase [22] have been described.

Oliver et al. [20] reported that the production of hemolysin may be significant in the fatal infection caused by *V. vulnificus*; and Stelma et al. [27] found that virulent *V. vulnificus* isolates produced high titers of hemolytic activity. Therefore, this suggested that a protein that possesses hemolytic activity might be a potential virulence factor. A pore-forming hemolysin (*vvhA*) is known to be cytolytic to Chinese hamster ovary (CHO) cells and to disrupt the membranes of various mammalian erythrocytes [11, 14, 31]. It also has the ability to enhance vascular permeability and is lethal to mice [11, 12, 17]. However, although correlations between hemolysin (*vvhA*) and the virulence of *V. vulnificus* strain have been reported as mentioned above, an isogenic mutant deficient in the production of hemolysin (*vvhA*) was still virulent in mice, as assayed with several animals [29]. We therefore reasoned that other hemolysins might be important for the pathogenesis of *V. vulnificus*.

In this study, we describe the cloning and expression of *hlyIII* gene from *V. vulnificus* that is able to induced hemolytic activity in *Escherichia coli*. Moreover, we also created a *hlyIII* knockout *V. vulnificus* strain that was found to be less virulent than the wild-type in mice.

Materials and Methods

Bacterial strains and plasmids. *Escherichia coli* HB101 (New England Biolabs, Beverly, MA) was used as host strain for the cloning experiment. *E. coli* S17-1λ*pir* was used as host strain for the conjugation experiment [13]. *V. vulnificus* CKM-1 is clinical isolate from the blood of a septicemic patient at the Hospital Center of National Cheng-Kung University [9]. *V. vulnificus* HLY-1, a *hlyIII* isogenic mutant, was derived from the parent strain CKM-1. Plasmid pUC19 (New England Biolabs) was used in the cloning experiment; plasmid pCVD442 [10] was used in the gene inactivation experiment. All strains were routinely grown in minimal medium (12.8 g/L $Na_2HPO_4 \cdot 7H_2O$, 3 g/L KH_2PO_4, 10 g/L NaCl, 1 g/L NH_4Cl, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, 4 g/L glucose) or Luria–Bertani (LB) medium at 37°C with aeration. Antibiotics were used as follows: ampicillin at 100 μg/mL for *E. coli*, and ampicillin at 100 μg/mL and rifampicin 50 μg/mL for *V. vulnificus*.

Molecular techniques. Standard techniques were used to construct

Correspondence to: C.L. Jeang; *email:* cljeang@nchu.edu.tw

recombinant plasmids [24]. DNA fragments used in cloning were extracted from agarose gels by using the Qiaex II kit (Qiagen, Mississaugua, Ontario, Canada). PCR was carried out according to the manufacturer's recommendations by using the *Taq* DNA polymerase kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Nucleotide sequence was determined by an autosequencer (ABI Prism 373 DNA Sequencer, Applied Biosystems).

Cloning of *Vibrio vulnificus hlyIII* gene. The complete coding sequence of *hlyIII* was cloned from the genomic library of CKM-1 strain [9] with a [α-^{32}P]-labeled DNA fragment of *hlyIII* by colony hybridization [24]. One positive plasmid (pYC4) was selected for DNA sequencing.

Expression of *hlyIII* in *E. coli*. A 1346 bp fragment containing *hlyIII* gene was amplified from pYC4 by PCR with the primers HF1 (5′-CGGGTACCATTGGTGATGCCAAAG-3′) and HR1 (5′-AAGAGCTCACCTTATTGGTGCTAT-3′). PCR product was digested with *Kpn*I and *Sac*I and inserted into identically digested pUC19. The resulting plasmid, pYC5, was introduced into calcium-competent *E. coli* HB101. Exponentially growing bacteria of *E. coli* HB101 bearing plasmid pYC5 or pUC19 were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 10 h at 37°C. The extract was separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue.

Hemolytic activity assay. Hemolytic activity assay was carried out according to the method described by Baida and Kuzmin [3].

Construction of *hlyIII* mutant. A 199 bp internal fragment of *hlyIII* gene of *V. vulnificus* CKM-1 was generated by PCR with primers HF2 (5′-CATGTCGACTAGCTGACCATTGCG-3′) and HR2 (5′-AAGGCATGCGCTAACTCACCAGC-3′). The PCR product was digested with *Sal*I and *Sph*I, and inserted into identically digested pCVD442. The resulting plasmid (pYC6) was transformed into *E. coli* S17-1λ*pir* and subsequently transferred into *V. vulnificus* CKM-1 via conjugation according to the method described by Hensel et al. [13] with minor modifications. Transconjugants were selected by ampicillin and rifampicin. The resultant strain HLY-1 was further confirmed by PCR and Southern blot analysis using *hlyIII* probe.

Reverse transcription-PCR (RT-PCR). SV total RNA isolation kit (Promega, Madison, WI) was used to extract RNA from wild-type CKM-1 and *hlyIII* mutant strains that had been grown in LB broth for 5 h at 37°C. Random hexamers were used to anneal to purified RNA (1 μg) for first-strain cDNA synthesis with Superscript II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR amplifications were performed with *Taq* DNA polymerase (Invitrogen) using an aliquot (1/10) of each RT reaction mixture as templates. *hlyIII* cDNA was amplified with primers HF2 (5′-CATGTCGACTAGCTGACCATTGCG-3′) and HR2 (5′-AAGGCATGCGCTAACTCACCAGC-3′), and 16S rRNA cDNA was amplified with primers UFUL (5′-GCCTAACACATGCAAGTCGA-3′) and URUL (5′-CGTATTACCGCGGCTGCTGG-3′) described by Nilsson et al. [19]. Thirty cycles of amplification were carried out, and each cycle consisted of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. RNA submitted to PCR without prior reverse transcription was used as negative control. 16S rRNA was used as internal control. PCR product was electrophoresed on 2% agarose gel and photographed under UV transillumination. DNA sequences of these amplified products were confirmed by DNA sequencing.

PCR. Chromosomal DNAs (100 ng/μL) were used as PCR templates. A pair of oligodeoxyribonucleotides (HF2 and HR2) were used as PCR primers. PCR was carried out as described above.

Virulence assay. BALB/c mice, 8 to 10 weeks old, purchased from the animal center of the College of Medicine at National Cheng-Kung University, were challenged by intraperitoneal injection of the bacterial suspension. A group of eight mice was given 0.2 mL of a 10-fold serially diluted bacterial suspension in phosphate-buffered saline per mouse and mortality was recorded 5 days postinfection. The doses lethal to 50% of the mice (LD_{50}) of each strain were calculated by the method of Reed and Muench [23].

Nucleotide sequence accession number. The nucleotide sequence of *hlyIII* of *V. vulnificus* has been assigned GenBank accession number AY293743.

Results and Discussion

Cloning of *V. vulnificus hlyIII* gene. During the sequencing of a clone containing a serum-resistant gene (*trkA*) of *V. vulnificus* CKM-1 [7], a partial DNA sequence, which exhibited homology to hemolysin III of *Bacillus cereus* [2], was noted. To isolate clones containing the complete coding sequence of hemolysin III (*hlyIII*) from *V. vulnificus*, a genomic library of CKM-1 DNA in the vector pBR322 was subjected to colony hybridization and probing with the radiolabeled C-terminal DNA fragment of *hlyIII* of *V. vulnificus*; several probe-reactive clones were isolated. A plasmid pYC4 containing a ~5 kb insert was selected for sequence analysis. Nucleotide sequence analysis predicted an open reading frame of 642 bp encoding a 214 amino acid polypeptide with a molecular mass of 23.5 kDa. Analysis of the deduced amino acid sequence of HlyIII for homology to known gene sequences in databases using the BLAST algorithm [1] was performed and several relevant sequences were identified. The amino acid sequence of HlyIII shared 48% sequence identity (69% similarity) to the hemolysin III from *B. cereus* (accession number P54176) [2] and 80% sequence identity to a putative hemolysin from *V. cholerae* (NP_229699). HlyIII also showed identities to putative hemolysins of other bacteria, including *Salmonella typhimurium* (71%; NP_461965.1), *Yersinia pestis* (66%; NP_404519.1), *Bacillus anthracis* (48%; NP_656106.1) and *Xanthomonas campestris* (47%; NP_638208.1).

Expression of the HlyIII protein in *E. coli* transformants. Expression of *hlyIII* upon induction with IPTG in *E. coli* HB101 carrying plasmid pYC5 is shown in Fig. 1. No major polypeptides could be detected in *E. coli* carrying only the pUC19 vector. The expressed *hlyIII* gene product migrated as a ~23 kDa polypeptide, which agreed well with the value calculated from the putative amino acid sequence.

Hemolytic activity. To further investigate the *hlyIII*

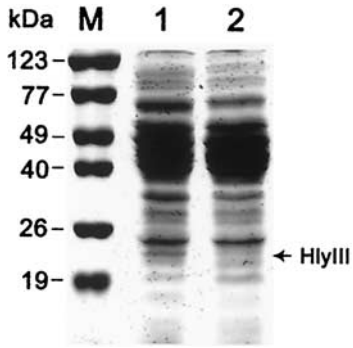

Fig. 1. Expression of *hlyIII* in *E. coli* HB101. Lane 1, crude extracts from *E. coli* HB101 carrying pYC5; lane 2, crude extracts from *E. coli* HB101 carrying pUC19; M, molecular weight markers.

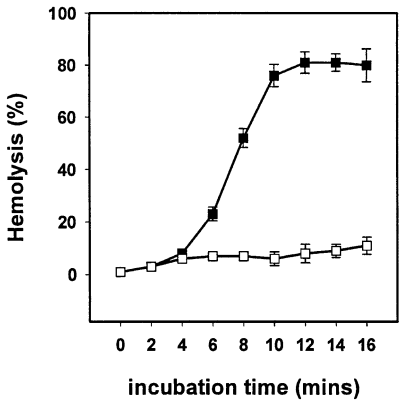

Fig. 2. Hemolysin (HlyIII)-induced hemolysis in a human erythrocyte suspension at 37°C. Crude proteins from *E. coli* containing pYC5 (filled symbols) or pUC19 (open symbols) were mixed with equal volumes of erythrocyte suspension and the mixtures incubated at 37°C for the indicated time periods. Percentage hemolysis was calculated as a ratio of A_{540} of hemolysis control. Data were expressed as the mean ± standard error of a representative experiment performed in duplicate.

gene encoding a protein responsible for hemolysis, the hemolytic activities of *E. coli* HB101 carrying pYC5 or pUC19 were compared. The HlyIII-induced hemolysis began after 3 min and was completed by 12 min of incubation at 37°C, but hemolytic activity was not observed in the negative control plasmid pUC19 (Fig. 2). These results showed that recombinant HlyIII exhibited hemolytic activity.

It has previously been reported that *B. cereus* hemolysin III acts as an oligomeric pore-forming hemolysin [3]. Since the putative amino acid sequence of *V. vulnificus* HlyIII showed 48% identity to hemolysin III of *B. cereus* [2], it is reasonable to conjecture that *V. vulnificus* HlyIII could also be a pore-forming hemolysin. However, additional work is needed to determine whether this is the case.

Isolation of the *hlyIII* mutant. To determine whether

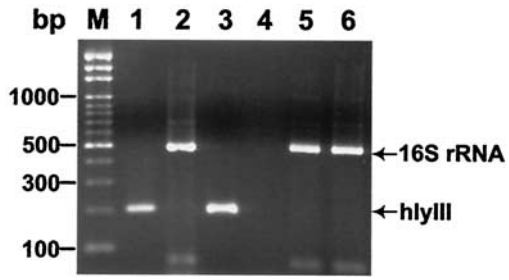

Fig. 3. Confirmation of the *V. vulnificus hlyIII* mutant by RT-PCR. RNAs isolated from *V. vulnificus* CKM-1 (lanes 3 and 5) and the *hlyIII* mutant (lanes 4 and 6) were used as template to generate cDNAs. Genomic DNA PCR was included as positive control (lanes 1 and 2). Primers HF2 and HR2, specific for *hlyIII*, produced in a band of 199 bp (lanes 1, 3 and 4); and primers UFUL and URUL, specific for 16S rRNA, resulted in a band of 492 bp (lanes 2, 5 and 6). The 16S rRNA served as an internal control for the presence of RNA used in each reaction.

the *hlyIII* gene is an important virulence factor in vivo, we created an insertional inactivation mutant of *hlyIII* in strain *V. vulnificus* CKM-1, as described in Materials and Methods. Insertional disruption of *hlyIII* gene in the HLY-1 strain was checked by PCR using a pair of primers complementary to sequences located in the *hlyIII* and ampicillin-resistant genes (data not shown). To determine whether expression of the *hlyIII* gene had been impaired, RT-PCR analysis of the parental and HLY-1 strains was performed. The results of RT-PCR yielded products of the expected sizes (199 bp) from the RNA of wild-type strain CKM-1 and that these amplified products were indeed the *hlyIII* was confirmed by DNA sequencing. In contrast, no amplification product was detected from the *hlyIII* mutant. The result of RT-PCR analysis confirmed the absence of HlyIII in the *hlyIII* mutant (Fig. 3). RNA submitted to PCR without prior reverse transcription was used as a negative control. No amplification product was observed in the negative control (data not shown).

On the other hand, the *hlyIII* mutant and wild-type showed identical growth rates under aerobic conditions in LB medium (data not shown). The result showed that *hlyIII* disruption did not appear to cause a growth defect in vitro. In addition, the HLY-1 strain had an opaque colony morphology and could grow in minimal medium similar to wild type CKM-1, suggesting that the *hlyIII* mutant HLY-1 probably was neither an unencapsulated mutant nor an auxotrophic mutant.

The hemolytic activities of the *V. vulnificus hlyIII* mutant and wild-type parent strain were compared on blood agar plates. No major differences between the two strains were observed after growth on 5% sheep blood agar plates. There are two possibilities to explain this phenomenon: (i) HlyIII in *V. vulnificus* is not expressed

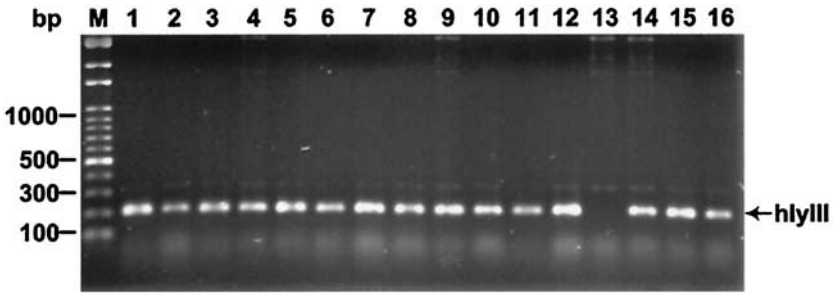

Fig. 4. Agarose gel electrophoresis of PCR products from different template DNAs. Lanes 1–11, *V. vulnificus* CKM-1 and 10 clinical isolates; lanes 12–16, five environmental isolates.

to a significant extent on blood agar plate and (ii) *V. vulnificus* may contain other proteins that can compensate for the loss of HlyIII. To our knowledge, *V. vulnificus* possesses at least two hemolytic proteins other than *hlyIII*. One (*vvhA*) is a pore-forming hemolysin [11, 14] and the other (*vllY*) exhibits sequence homology to 4-hydroxyphenylpyruvate dioxygenase family proteins [6]. This may explain why the level of hemolysis is similar between the wild-type and *hlyIII* mutant on blood agar plates.

Virulence in mice. The virulence of the *hlyIII* isogenic mutant HLY-1 and wild-type strain CKM-1 were studied by intraperitoneal infection of BALB/c mice. The LD_{50} value of the mutant HLY-1 in normal mice was 8.0×10^6 at 72 h. This was a 16-fold increase compared with the LD_{50} value of 5.0×10^5 for the wild-type in mouse.

Conservation of the *hlyIII* gene among clinical *V. vulnificus* isolates. To elucidate whether *hlyIII* is common to clinical *V. vulnificus* isolates, a pair of PCR primers (HF2 and HR2) were designed to amplify a 199 bp region within the ORF of *hlyIII*. The test set of 16 *V. vulnificus* isolates included 11 clinical isolates and five environmental isolates. All the isolates, except one environmental isolate, produced an amplification product (Fig. 4). This result demonstrates that *hlyIII* could potentially play a role in pathogenicity.

A variety of endotoxins and exotoxins have been implicated as putative virulence factors for *V. vulnificus*, including polysaccharide capsules [26, 28, 32], metalloprotease [16], and cytolysin [11]. CPS has been shown to play a role in mouse virulence in *V. vulnificus* [26, 32]. Opaque-to-translucent colony variations are associated with CPS production. Opaque colonies are encapsulated while translucent colonies have little or no capsule production [32]. Since the *hlyIII* mutant exhibited an opaque colony phenotype on LB agar, it is likely that the mouse virulence of *hlyIII* mutant was due to the loss of HlyIII but not CPS. However, because it is known that some *V. vulnificus* isolates that express less CPS still form opaque colonies on LB agar [30], at present we cannot exclude the possibility that the *hlyIII* mutant produces less CPS than wild-type strain; this remains to be determined. No exotoxin has previously been identified as a virulence factor in an animal model by gene disruption. In this study, we identified HlyIII of *V. vulnificus* as a virulence factor in mice, although it has not yet been investigated whether HlyIII is an exotoxin during infection.

In summary, we showed that a recombinant hemolysin (*hlyIII*) produced from *E. coli* exhibits hemolytic activity. In addition, we also created an isogenic insertionally inactivated *hlyIII* mutant that exhibited attenuated virulence in mice compared with the wild-type strain. This is the first study to report the relationship between HlyIII and the virulence of *V. vulnificus* in mice. Additional studies are needed to investigate the role of HlyIII in pathogenesis during infection with *V. vulnificus* in vivo.

ACKNOWLEDGMENTS

We gratefully acknowledge M.S. Donnenberg and J.B. Kaper for providing their plasmid.

Literature Cited

1. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
2. Baida GE, Kuzmin NP (1995) Cloning and primary structure of a new hemolysin gene from *Bacillus cereus*. Biochim Biophys Acta 1264:151–154
3. Baida GE, Kuzmin NP (1996) Mechanism of action of hemolysin III from *Bacillus cereus*. Biochim Biophys Acta 1284:122–124
4. Bisharat N, Agmon V, Finkelstein R, Raz R, Ben-Dror G, Lerner L, et al. (1999) Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel. Lancet 354:1421–1424
5. Blake PA, Merson MH, Weaver RE, Hollis DG, Heublein PC (1979) Disease caused by a marine *Vibrio*: Clinical characteristics and epidemiology. N Engl J Med 300:1–5
6. Chang TM, Chuang YC, Su JH, Chang MC (1997) Cloning and sequence analysis of a novel hemolysin gene (*vllY*) from *Vibrio vulnificus*. Appl Environ Microbiol 63:3851–3857
7. Chen YC, Chuang YC, Chang CC, Jeang CL, Chang MC (2004) A K^+ uptake protein, TrkA, is required for serum, protamine, and polymyxin B resistance in *Vibrio vulnificus*. Infect Immun 72:629–636

8. Chuang YC, Yuan CY, Liu CY, Lan CK, Huang AH (1992) *Vibrio vulnificus* infection in Taiwan: Report of 28 cases and review of clinical manifestations and treatment. Clin Infect Dis 15:271–276
9. Chuang YC, Chang TM, Chang MC (1997) Cloning and characterization of the gene (*empV*) encoding extracellular metalloprotease from *Vibrio vulnificus*. Gene 189:163–168
10. Donnenberg MS, Kaper JB (1988) Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect Immun 59:4310–4317
11. Gray LD, Kreger AS (1986) Purification and characterization of an extracellular cytolysin produced by *Vibrio vulnificus*. Infect Immun 48:62–72
12. Gray LD, Kreger AS (1987) Mouse skin damage caused by cytolysin from *Vibrio vulnificus* and by *V. vulnificus* infection. J Infect Dis 155:236–241
13. Hensel M, Shea JE, Gleeson C, Jones MD, Dalton E, Holden DW (1995) Simultaneous identification of bacterial virulence genes by negative selection. Science 269:400–403
14. Kim HR, Rho HW, Jeong MH, Park JW, Kim JS, Park BH, et al. (1993) Hemolytic mechanism of cytolysin produced from *V. vulnificus*. Life Sci 53:571–577
15. Klontz KC, Lieb S, Schreiber M, Janowski HT, Baldy LM, Gunn RA (1988) Syndromes of *Vibrio vulnificus* infections: Clinical and epidemiologic features in Florida cases, 1981–1987. Ann Intern Med 109: 318–323
16. Kothary MH, Kreger AS (1987) Purification and characterization of an elastolytic protease of *Vibrio vulnificus*. J Gen Microbiol 133:1783–1791
17. Kreger A, Lockwood D (1981) Detection of extracellular toxin(s) produced by *Vibrio vulnificus*. Infect Immun 33:583–90
18. Litwin CM, Rayback TW, Skinner J (1996) Role of catechol siderophore synthesis in *Vibrio vulnificus* virulence. Infect Immun 64:2834–2838
19. Nilsson WB, Paranjype RN, DePaola A, Strom MS (2003) Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. J Clin Microbiol 41:442–446
20. Oliver JD, Wear JE, Thomas MB, Warner M, Linder K (1986) Production of extracellular enzymes and cytotoxicity by *Vibrio vulnificus*. Diagn Microbiol Infect Dis 5:99–111
21. Paik KW, Moon B, Park CW, Kim KT, Ji MS, Choi SK, et al. (1995) Clinical characteristics of ninety-two cases of *Vibrio vulnificus* infections. Kor J Infect Dis 27:355–365
22. Paranjpye RN, Lara JC, Pepe JC, Pepe CM, Strom MS (1998) The type IV leader peptidase/N-methyltransferase of *Vibrio vulnificus* controls factors required for adherence to Hep-2 cells and virulence in iron-overloaded mice. Infect Immun 66:5659–5668
23. Reed LJ, Muench H (1938) A simple method of estimating the 50% endpoints. Am J Hyg 27:493–497
24. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
25. Shapiro RL, Altekruse S, Hutwagner L, Bishop R, Hammond R, Wilson S, et al., the Vibrio Working Group (1998) The role of gulf coast oysters harvested in warmer months in *Vibrio vulnificus* infections in the United States, 1988–1996. J Infect Dis 178:752–759
26. Simpson LM, White VK, Zane SF, Oliver JD (1987) Correction between virulence and colony morphology in *Vibrio vulnificus*. Infect Immun 55:269–272
27. Stelma GN Jr, Reyes AL, Peeler JT, Johnson CH, Spaulding PL (1992) Virulence characteristics of clinical and environmental isolates of *Vibrio vulnificus*. Appl Environ Microbiol 58:2776–2782
28. Wright AC, Simpson LM, Oliver JD, Morris JG Jr (1990) Phenotypic evaluation of acapsular transposon mutants of *Vibrio vulnificus*. Infect Immun 58:1769–1773
29. Wright AC, Morris JG Jr (1991) The extracellular cytolysin of *Vibrio vulnificus*: Inactivation and relationship to virulence in mice. Infect Immun 59:192–197
30. Wright AC, Powell JL, Tanner MK, Ensor LA, Karpas AB, Morris JG Jr, et al. (1999) Differential expression of *Vibrio vulnificus* capsular polysaccharide. Infect Immun 67:2250–2257
31. Yamamoto K, Wright AC, Kaper JB, Morris JG Jr (1990) The cytolysin gene of *Vibrio vulnificus*: Sequence and relationship to the *Vibrio cholerae* E1 Tor hemolysin gene. Infect Immun 58: 2706–2709
32. Yoshida S, Ogawa M, Mizuguchi Y (1985) Relation of capsular materials and colony opacity to virulence of *Vibrio vulnificus*. Infect Immun 47:446–451