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Characteristics and Pathogenicity for Japnaes Eel *Anguilla japonica* of *Vibrio vulnificus* Isolated from Oyster, Sediment and Seawater in the Korea Coast

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Abstract

Biotyping of *Vibrio vulnificus* strains isolated from marine environments along the south coast of Korea showed that the majority of the isolates (94.7%) belonged to biotype 1 and the remaining isolates (5.3%) belonged to biotype 2. Analysis of 16S rRNA *V. vulnificus* strains isolated from marine environments using a multiplex polymerase chain reaction (PCR) revealed that 78.7% were type A and 21.3% were type B. Random amplified polymorphic DNA (RAPD) was used to analyze the genomic differences in *V. vulnificus* among the biotype 2 strains isolated from marine environments (newly isolated strains group) and reference strains obtained from infected eels (reference strains group). The two groups had distinctly different profiles of the amplicons produced from RAPD. Additionally, biochemical comparison of these strains revealed that all four strains isolated from marine environments differed from the strains isolated from eels in their ability to promote D-mannitol fermentation. Two (NH 1 and NH 2) out of four isolates of biotype 2 from marine environments showed pathogenicity in eels *Anguilla japonica* in a challenge test. These isolates did not agglutinate with antisera against *V. vulnificus* NCIMB 2137 (serovar E), ATCC 27562 (non-serovar E), and ATCC 33816 (atypical serovar E).

Key words: *Vibrio vulnificus*, *Anguilla japonica*, Biotype, Genotype, RAPD, Japanes eel, Pathogenicity

Introduction

Vibrio vulnificus is a Gram negative bacterium belonging to *Vibrionaceae*. It is widely distributed in fresh water and seawater (Strom and Paranjpye, 2000) and has been isolated from oysters, shellfish, fish, sediment, and plankton (Oliver et al., 1983; Wright et al., 1993; DePaola et al., 1994). Sepsis in humans due to *V. vulnificus* infection or consumption of contaminated fish and shellfish (Linkous and Oliver, 1999) results in mortality rates of at least 60% (Doly, 1989). *V. vulnificus* is therefore a significant pathogen threatening the safety of seafood.

V. vulnificus is divided into three biotypes based on biochemical and genetic characteristics and pathogenicity. *V. vulnificus* biotype 1 is responsible for 95% of deaths due to the consumption of contaminated shellfish (Oliver et al., 1991). Biotype 3 has only been isolated in Israel (Bisharat et al., 1999) and shows a regional distribution. Biotype 2 was first isolated from cultured Japanese eels in 1982 (Tison et al., 1982) and in addition to eels is known to also cause sepsis in humans (Amaro and Biosca, 1996). Biotype 2 is divided into four serovars (Fouz et al., 2006). Most biotype 2 strains

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are isolated from eels and have the same serotype, serovar E (Dalsgaard et al., 1996). However, in recent years, it was reported that serovar A strains belonging to biotype 2 are also pathogenic to eels (Fouz et al., 2006). Biotype 2 strains that cause infection in eels are carried in water (Noales et al., 2001) and can survive more than 50 days in seawater (Biosca et al., 1996). *V. vulnificus* biotype 2 strains have been isolated from marine environments in South Korea, Taiwan, and Denmark (Hor et al., 1995; Høi et al., 1998; Kim and Jeong, 2001), but their characteristics, such as phenotype, genotype, and serotype, are unknown. It was reported that the survival and distribution of *V. vulnificus* are affected by water temperature and salinity (Kaysner et al., 1987; Wright et al., 1996), hence the biotype 2 strains isolated from eels and from seawater may differ, but there have been no studies comparing them.

V. vulnificus is divided into type A and type B based on differences in the nucleotide sequences of 16S rRNA (Aznar et al., 1994), and pathogenicity in humans is associated with type B (Nilsson et al., 2003). However, there is little information on the distribution and characteristics of *V. vulnificus* 16S rRNA isolated from marine environments.

We analyzed the distribution characteristics by biotype and genotype of *V. vulnificus* from the Korean coast, and the biochemical, serological, and genetic characteristics of *V. vulnificus* biotype 2 strains from these marine environments were compared with those of biotype 2 strains from eels.

Materials and Methods

V. vulnificus isolation

V. vulnificus was isolated from seawater, sediment, and oysters collected from the southern coast of Korea (at 34.950°N, 127.858°E and 34.830°N, 127.926°E) in August, 2003. The seawater temperature was 25°C and the salinity was 32 ppt. Seawater was collected from the surface layer and 100 μL volumes were inoculated in modified cellobiose-polymyxin B-colistin (mCPC) agar. The collected sediment and oysters were homogenized with sterilized phosphate buffered saline (PBS), inoculated in mCPC agar, and subsequently incubated for 24 h at 40°C. The yellow colonies of bacteria formed in the mCPC agar were identified as *V. vulnificus* by polymerase chain reaction (PCR).

Bacterial strains used

The fourteen strains used in this study included ten reference strains received from the Korean Collection for Type Culture (KCTC), Pukyong National University (PKNU), American Type Culture Collection (ATCC) and four newly isolated strains from marine environments (Table 1). All strains were used for analysis after incubation at 37°C for 24 h using 1% (w/v) sodium chloride enriched tryptic soy broth (TSB) or tryptic soy agar (TSA; BD, Detroit, MI, USA), and were stored at -80°C with 20% (w/v) glycerol.

Molecular assays

PCR and 16S rRNA sequencing

For the identification of *V. vulnificus*, Cyt 1 (5′-ACAAAGACGGCCGCAAAGTGG-3′) and Cyt 2 (5′-AGCCCGCAGAGCCGTAAACC-3′) primers, which were designed using the cytotoxin-hemolysin gene as a template, and Vib 1 (5′-GTGGTAGTGTTAATAGCACT-3′), Vib 2 (5′-TCTAGCGGAGACGCTGGA-3′), and Vib 3R (5′-GCTCACTTTCGCAAGTTGGCC-3′) primers (Kim and Jeong, 2001), which can be used to identify *V. vulnificus* and simultaneously classify the 16S rRNA type, were used. All primers were synthesized using an automated DNA synthesizer (Bioneer, Daejeon,

Table 1. Biotype and 16S rRNA type of *Vibrio vulnificus* used in this study

Strain name	Biological origin	Geographic origin	biotype	16S rRNA type	Source
Reference strains group					
ATCC 27562	human blood	U.S.A.	1	A	PKNU
ATCC 33816	human blood	U.S.A.	1	B	ATCC
CJVV O4	human blood	Korea	1	B	PKNU
ATCC 33147 (= KCTC 2983)	eel	Japan	2	A	KCTC
ATCC 33148 (= KCTC 2985)	eel	Japan	2	A	KCTC
ATCC 33149	eel	Japan	2	A	PKNU
CECT 4607 (= KCTC 2988)	eel	Spain	2	A	KCTC
ES 7602	eel	Japan	2	A	PKNU
LMG 16868 (= KCTC 2982)	eel	Spain	2	A	KCTC
NCIMB 2137 (= KCTC 2984)	eel	Japan	2	A	KCTC
Newly isolated strains group					
HD 1	oyster	Korea	2	B	This work
HD 2	seawater	Korea	2	B	This work
NH 1	seawater	Korea	2	B	This work
NH 2	seawater	Korea	2	B	This work

Korea). One pmol of each primer and isolated DNA were mixed with PCR premix (Bioneer) and PCR amplification was performed using a PTC–220 DNA Engine Dyad Peltier thermal cycler (Bio-Rad, Hercules, CA, USA). In the PCR cycle, pre-PCR was at 94°C for 5 min, denaturation was at 94°C for 0.5 min, annealing was at 55°C for 0.5 min, and extension was at 72°C for 0.5 min (30 cycles), with a final extension at 72°C for 7 min. The results were confirmed by electrophoresis at 100 V for 20 min in 1.0% TAE agarose (with 0.5 μg/mL ethidium bromide (EtBr)) and 0.5 x TAE buffer (with 0.5 μg/mL EtBr). PCR was performed on *V. vulnificus* biotype 2 (Sanjuan and Amaro, 2007) using the SerE-R (5′-CGCGCTTAGATTTGTCTCACC-3′) and SerE-F (5′-TGTTGTTCTTGCCCACTCTC-3′) primers to identify *V. vulnificus* serovar E. 16S rRNA of *V. vulnificus* biotype 2 strains showing a negative reaction in an indole test were amplified by PCR, cloning was conducted using a TOPO A cloning kit (Invitrogen, Carlsbad, CA, USA), and the results were compared with the GeneBank database after sequencing.

Random amplified polymorphic DNA (RAPD)

R1 primer (5′-GCCACTTATG-3′) was used for RAPD analysis. 1 pmol of primer was mixed with PCR premix (Bioneer) for PCR amplification, with pre-PCR at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 37°C for 1 min, and extension at 72°C for 1 min (45 cycles), with a final extension at 72°C for 10 min. The results were confirmed by electrophoresis at 100 V for 30 min in 2.0% TAE agarose (with 0.5 μg/mL EtBr) and 0.5× TAE buffer (with 0.5 μg/mL EtBr). In the RAPD electrophoretic products, we denoted a matching band as 1 and a mismatching band as 0. Cluster analysis was performed using the unweighted pair-group method using arithmetic averages (UPGMA) in TREECON software version 1.3b (Van de Peer and De Wacher, 1994).

Biochemical and slide agglutination test

Biochemical characteristics were analyzed using an API 20E kit (BioMerieux, Paris, France), following the manufacturer's instructions. The serological characteristics of the isolates were identified using a slide agglutination test and rabbit antisera against *V. vulnificus* ATCC 27562, ATCC 33816, NCIMB 2137, and NH 1.

Pathogenicity in eels

V. vulnificus cultured in TSB at 37°C for 18 h was diluted with sterile PBS to obtain an absorbance value of 1.0 at 600 nm and the the number of bacteria was counted using the viable counting method. Eels (mean body weight, 12.5 g) were anesthetized with MS–222 (Sigma-Aldrich, USA), and 0.1 mL of diluted bacteria was injected intraperitoneally. The cumulative mortality was checked for 7 days in 5 ppt artificial seawater. *V. vulnificus* was isolated from the kidneys of dead fish.

Viability of *V. vulnificus* in eel serum

The number of viable *V. vulnificus* in eel serum was recorded using the method of Amaro et al. (1999). Blood was collected from four eels and centrifuged at 8,000 rpm for 10 min to separate the serum. The serum was stored at -80°C until analysis and the cultured bacteria were diluted with saline solution, mixed with the serum to a concentration of 2×10^5–10^6 CFU/mL, and stored at 25°C. The number of bacteria was counted at 0, 1, 2, 4, and 6 h. A 25 μL diluted sample was added to TSA and the number of bacteria counted after incubation at 25°C for 24 h.

Results

V. vulnificus isolation from marine environments

V. vulnificus was isolated from seawater, sediment, and oysters. Among the yellow colonies formed in mCPC agar, 75 strains produced at least 408 bp of the expected product from PCR using Cyt 1 and Cyt 2 primers, and also produced 273 bp of product using Vib 1, Vib 2, and Vib 3R primers. Hence, they were identified as *V. vulnificus*. Among the 75 strains of *V. vulnificus,* 30 strains were isolated from oysters (40.0%), 27 strains from seawater (36.0%), and 18 strains from sediment (24.0%). Biotype 1 constituted 71 strains (94.7%) and biotype 2 four strains (5.3%; Table 2). Of the four strains of biotype 2, three strains from seawater and one strain from oysters were isolated, and 16S rRNA analysis revealed 99% nucleotide similarity to *V. vulnificus* (accession number NR074889).

Table 2. Characteristics of *Vibrio vulnificus* isolates

Samples	biotype[1]		16S rRNA type[2]	
	1	2	A	B
Oyster	29	1	21	9
Sediment	18	0	17	1
Seawater	24	3	21	6
Total	71	4	59	16

[1]Determined by indole test in trypton broth
[2]Determined by tri-primer PCR gene amplification

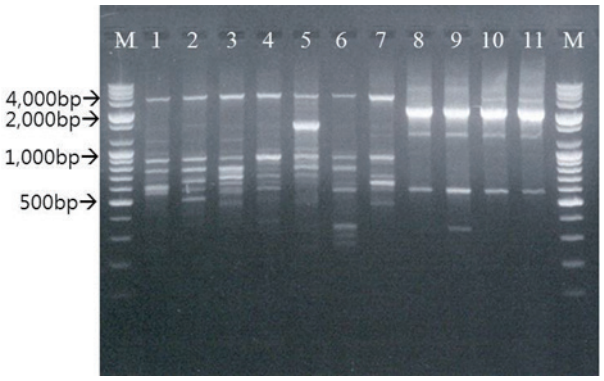

Fig. 1. RAPD amplification patterns of *Vibrio vulnificus* using R1 primer. Lanes: M, DNA size standards; 1, *V. vulnificus* ES7602; 2, LMG 16868; 3, ATCC 33147; 4, ATCC 33148; 5, CECT 4607; 6, NCIMB 2137; 7, ATCC 33149; 8, NH 1; 9, NH 2; 10, HD 1; 11, HD 2. Seven strains of lane 1 to lane 7 were isolated from eels and four strains of lane 8 to lane 11 were isolated from marine environments.

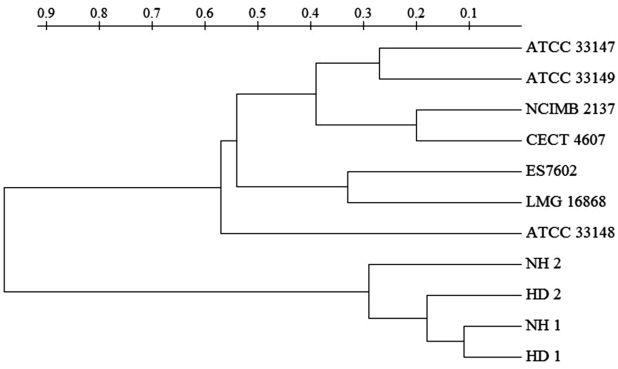

Fig. 2. UPGMA dendrogram of *Vibrio vulnificus* biotype 2 isolated from eel and marine environments using the TREECON program. The scale indicates dissimilarity.

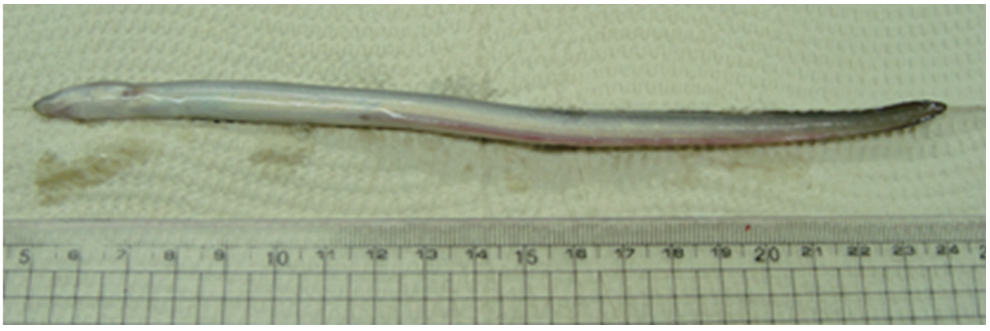

Fig. 3. External signs on the moribund eel *Anguilla japonica* infected by i.p. challenge with the *Vibrio vulnificus* biotype 2 strain NH 1 (2×10^6 CFU/fish).

16S rRNA type classification

Among the 75 strains selected by PCR to classify the 16S rRNA type of *V. vulnificus*, 59 strains (78.7%) produced 2 amplicons, 273 bp and 826 bp, and 16 strains (21.3%) produced 273 bp of one amplicon; hence, they were classified as type A and type B, respectively (Table 2). Four *V. vulnificus* biotype 2 strains from seawater, NH 1, NH 2, HD 1, and HD 2, were identified as 16S rRNA type B, and *V. vulnificus* ATCC 33147, ATCC 33148, ATCC 33149, CECT 4607, ES 7602, LMG 2982, and NCIMB 2137 from eels were identified as 16S rRNA type A. *V. vulnificus* biotype 1 strain ATCC 27562 was type A and the remaining strains, ATCC 33816 and CJVV O4, were type B (Table 1).

Classification by RAPD

Biotype 2 strains were divided into two groups, and strains from eels and those from marine environments were distinguished by their RAPD patterns (Figs. 1, 2).

Biochemical characteristics of *V. vulnificus*

We examined the biochemical characteristics of nine *V. vulnificus* reference strains and four newly isolated strains from seawater using an API 20E kit (Table 3). Among the 21 items from the API 20E kit, all 13 strains showed the same results in 16 items, and six items showed varied results. All strains were positive for four items, LDC, GEL, GLU, and OX, and negative for 12 items, ADH, CIT, H_2S, URE, TDA, VP, INO, SOR, RHA, SAC, MEL, and ARA. ONPG, ODC, IND, MAN, and AMY differed among the strains. Only NCIMB 2137 was negative in ONPG. All biotype 2 strains, but no biotype 1 strain, were negative for IND. For MAN, biotype 2 *V. vulnificus* NH 1, NH 2, HD 1, and HD 2 and biotype 1 *V. vulnificus* ATCC 33816 and CJVV O4 were positive.

Pathogenicity in eels

V. vulnificus biotype 2 from marine environments was used to artificially infect eels to investigate its pathogenicity. The

50% lethal doses (LD_{50}) of NH 1 and NH 2 from seawater for eels with a mean body weight of 12.5 g were 6.5×10^5 CFU/fish and 1.3×10^8 CFU/fish, respectively, whereas HD 1 and HD 2 were not pathogenic at a dose of 2×10^8 CFU/fish. The biotype 2 *V. vulnificus* strains ES 7602 and NCIMB 2137 from eels that were used as controls were pathogenic at a LD_{50} of 9.3×10^6 CFU/fish and 7.8×10^5 CFU/fish, respectively, but the biotype 1 strains ATCC 27562 and CJVV O4 were not pathogenic even at a dose of 2×10^8 CFU/fish. Dead eels infected with *V. vulnificus* showed symptoms of congestion in the fin base (Fig. 3).

Agglutination reactions

The rabbit antiserum against *V. vulnificus* NCIMB 2137, ATCC 27562, and ATCC 33816 did not display any cross-reaction with four *V. vulnificus* biotype 2 strains isolated in this study, whereas the antiserum against *V. vulnificus* NH 1 displayed strong cross-reaction.

Viability of *V. vulnificus* in eel serum

In the case of biotype 1, the viability of ATCC 27562 and CJVV O4 was less than 0.1%. In the case of biotype 2, the numbers of ATCC 33147, ES 7602, and NH 1 increased 344–3,569%, NH 2 showed a decrease to 97%, while HD 1 and HD 2 decreased to 7.8% and 6.8%, respectively (Fig. 4).

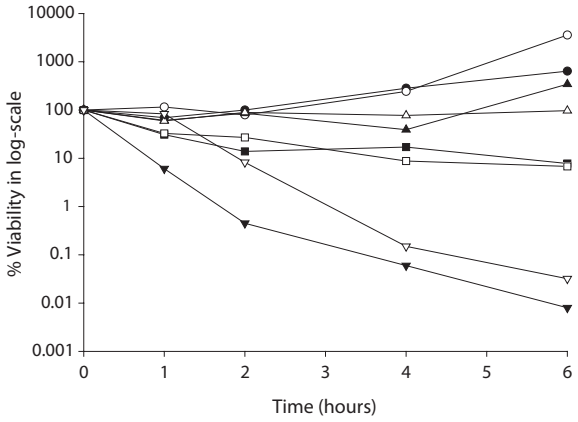

Fig. 4. The number of viable *Vibrio vulnificus* strains for 6 h of incubation in fresh eel serum. ●, *V. vulnificus* ES 7602; ○, ATCC 33147; ▲, NH 1; △, NH 2; ■, HD 1; □, HD 2; ▽, CJVV 04

Discussion

V. vulnificus has been isolated from locations around the world, including in Japan, Taiwan, South Korea, Denmark, and Spain, and has also been isolated from seawater, oysters, Manila clams, sea cucumbers, and eels. *V. vulnificus* is divided into 3 biotypes, and biotype 2 is considered to be a zoonotic pathogen because it is pathogenic to humans and eels.

Table 3. Biochemical characteristics of *Vibrio vulnificus* determined by API 20E kit

	Reference strains group										Newly isolated strains group			
	Biotype 1			Biotype 2							Biotype 2			
	ATCC 27562	ATCC 33816	CJVV O4	ATCC 33147	ATCC 33148	ATCC 33149	CECT 4607	ES 7602	LMG 16868	NCIMB 2137	HD 1	HD 2	NH 1	NH 2
ONPG	+	+	+	+	+	+	-	+	+	+	+	+	+	+
ADH	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LDC	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ODC	+	-	+	-	-	-	+	-	-	-	+	+	+	+
CIT	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-
URE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TDA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IND	+	+	+	-	-	-	-	-	-	-	-	-	-	-
VP	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GEL	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GLU	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MAN	-	+	+	-	-	-	-	-	-	-	+	+	+	+
INO	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SOR	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RHA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAC	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MEL	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMY	+	+	+	-	-	+	+	-	+	+	+	+	+	+
ARA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OX	+	+	+	+	+	+	+	+	+	+	+	+	+	+

In this study, the proportion of *V. vulnificus* biotype 2 isolated from seawater, oysters, and sediment was 5.3%, which is similar to the finding that three out of 40 strains (7.5%) from the southern coast of South Korea were biotype 2 (Kim and Jeong, 2001). The proportion of biotype 2 among the total *V. vulnificus* in the marine environment is unknown, but a biotype 2 ratio of 0.4% was reported among 706 strains from seawater off the coast of Denmark (Høi et al., 1998), and ratios of 91% for biotype 1 among 77 strains of *V. vulnificus* were reported from Taiwan seawater and 2% for biotype 2 (Hor et al., 1995). Hence, the proportion of *V. vulnificus* biotype 2 isolated from the Korean marine environment is higher than that from Denmark and lower than that from Taiwan.

The *V. vulnificus* genotype is closely related to pathogenicity because 26 strains among 34 strains from humans (76.5%) were 16S rRNA type B, but only two strains among 33 strains from oysters and seawater (6.1%) were type B (Nilsson et al., 2003). In this study, 75 *V. vulnificus* strains from the Korean marine environment were divided into 59 strains of 16S rRNA type A (78.7%) and 16 strains of type B (21.3%), which is fewer type B strains than the 65% among 40 strains reported by Kim and Jeong (2001). The proportion of *V. vulnificus* genotype is associated with temperature. The 16S rRNA type B *V. vulnificus* (C genotype) proportion in North Carolina was 29% during low-temperature periods and over 60% from August to October when the water temperature is approximately 30°C (Warner et al., 2008). Thus, the distribution of *V. vulnificus* genotype can vary depending on the time of the study. In addition, the proportion of type B in our study was greater than the 6.1% reported by Nilsson et al. (2003), but was similar to the value reported by Tao et al. (2012) of 29% in the Gulf of Mexico; hence, it is thought that the geographic distribution of *V. vulnificus* differs depending on the genotype. It is known that some forms of *V. vulnificus* biotype 2 are pathogenic to eels, but the biotype 2 isolated from Taiwanese oysters and seawater is not pathogenic to eels (Amaro et al., 1999); hence, the biotype 2 isolated from the marine environment differs from that in eels. HD 1 and HD 2 isolated in this study were not pathogenic to eels, which is consistent with the study by Amaro et al. (1999); however, NH 1 and NH 2 were pathogenic to eels and were elevated in eel serum. This indicates that NH1 and NH2 have similar pathogenicity and resistance to eel serum as biotype 2 strains from eels. The *V. vulnificus* strains, ES 7602 and ATCC 33147, which have high pathogenicity in eels, were elevated in eel serum; hence, the viability in eel serum appears to be related to pathogenicity. NH1 is pathogenic to eels, but when we artificially infected flatfish, which is commonly cultivated in Korea, no dead fish or symptoms were observed (data not shown), which indicates that NH1 is species-specific.

The biotype 2 strains isolated from eels and the biotype 2 strains isolated in this study differed in their biochemical characteristics, genotype and serotype. Four biotype 2 strains from seawater were all 16S rRNA type B, but the *V. vulnificus* biotype 2 strains from eels were all type A. In the results from RAPD, 16S rRNA type A and B showed different tendencies, which confirmed that the biotype 2 from eels and seawater are genetically distinct. Four biotype 2 strains isolated from seawater and oysters could ferment D-mannitol. Thus, they differ from the strains isolated from eels and eel farms, which cannot ferment D-mannitol (Biosca et al., 1996; Amaro et al., 1999; Fouz et al., 2007). Among the biotype 1 strains, 16S rRNA type B, *V. vulnificus* CJVV O4 was able to ferment D-mannitol, but type A, ATCC 27562 could not ferment D-mannitol, and several *V. vulnificus* biotype 1 strains showed similar results (data not shown); hence, we can distinguish the 16S rRNA type of *V. vulnificus* through D-mannitol fermentation ability.

In this study, agglutination between four biotype 2 strains isolated from marine environments and rabbit antiserum against *V. vulnificus* NCIMB 2137, known as serovar E, did not occur, and the strains were not serovar E because PCR results were negative (Sanjuan and Amaro, 2007). In addition, *V. vulnificus* ATCC 27562, a non-serovar E, displayed no agglutination with rabbit antiserum and no agglutination was seen with *V. vulnificus* ATCC 33816, an atypical serovar E (Marco-Noales et al., 2000), thus the strains do not belong to serovar E, non-serovar E, or atypical serovar E. There have been recent studies on *V. vulnificus* serovar A, but no comparison of serotype to serovar A was performed in this study. However, *V. vulnificus* serovar A is able to produce indole. Thus, it differs from the biotype 2 strains isolated from marine environments in this study.

V. vulnificus biotype 2 strains isolated from seawater and oysters differed from the biotype 2 strains isolated from eels in phenotype, genotype, and serotype, and by their ability to ferment D-mannitol. In addition, they were all of 16S rRNA type B, and the newly isolated *V. vulnificus* biotype 2 strains did not belong to serovar E.

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