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Evaluation of Genotypic and Phenotypic Methods To Distinguish Clinical from Environmental *Vibrio vulnificus* Strains▽†

Eva Sanjuán,[1]‡ Belén Fouz,[1] James D. Oliver,[2] and Carmen Amaro[1]*

Departamento de Microbiología y Ecología, Universidad de Valencia, Avda. Dr. Moliner 50, 46100 Burjassot, Valencia, Spain,[1] and Department of Biology, University of North Carolina at Charlotte, Charlotte, North Carolina 28223[2]

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Vibrio vulnificus **is a heterogeneous bacterial species that comprises virulent and avirulent strains from environmental and clinical sources that have been grouped into three biotypes. To validate the typing methods proposed to distinguish clinical from environmental isolates, we performed phenotypic (API 20E, API 20NE, and BIOLOG tests) and genetic (ribotyping and DNA polymorphism at several loci) studies with a large strain collection representing different biotypes, origins, and host ranges. No phenotypic method was useful for biotyping or grouping strains with regard to the origin of an isolate, and only the BIOLOG system was reliable for identifying the strains at the species level. DNA polymorphisms divided the population into three major profiles. Profile 1 strains were *vcg* type C, 16S rRNA type B, and *vvh* type 1 and included most of the biotype 1 human septicemic isolates; profile 2 strains were *vcg* type E, 16S rRNA type A, and *vvh* type 2 and included all biotype 2 isolates together with biotype 1 isolates from fish and water and some human isolates; and profile 3 strains were *vcg* type E, 16S rRNA type AB, and *vvh* type 2 and included biotype 3 strains. Ribotyping divided the species into two groups: one group that included profile 1 biotype 1 isolates and one group that included isolates of all three biotypes with the three profiles described above. In conclusion, no genotyping system was able to distinguish either clinical strains from environmental strains or biogroups within the species *V. vulnificus*, which suggests that new typing methodologies useful for public health have to be developed for this species.**

Vibrio vulnificus is an aquatic bacterial species that produces infections in fish and humans (30, 31, 36). Although human infections are relatively uncommon, they can be life threatening in patients with chronic or immunocompromising diseases (32, 36). The main transmission routes of human vibriosis are consumption of raw or undercooked shellfish and exposure of open wounds or sores to seawater (32, 36). The mortality rate due to primary septicemia after contaminated shellfish consumption is approximately 50%, and the mortality rate resulting from reported wound infections is 25% (4, 20).

This species is phenotypically and serologically heterogeneous (9, 17, 38). Originally, it was divided into two biotypes, one virulent for humans and one virulent for fish (38). In early studies, negative results for indole production, ornithine decarboxylase activity, acid production from mannitol and sorbitol, and growth at 42°C, as well as serological specificity, allowed investigators to distinguish the first fish isolates (biotype 2 serovar E) from human isolates (9, 38). However, this simple scheme of intraspecific classification lost its utility when more strains were isolated from fish vibriosis worldwide (19, 21, 24). These new isolates differed serologically and phenotypically from the isolates initially studied (21, 24) and were grouped in two additional serovars (serovars A and I) using the same serotyping system (21; C. Amaro, unpublished results). Interestingly, biotype 2 serovar E was also isolated from human infections, usually after manipulation of diseased fish, which increased the diversity of isolates able to infect humans (1). In addition to this biotype 2 heterogeneity, a third biotype was described in 1999 in Israel (11). To date, this biotype includes only isolates from wound infections initiated by handling spiny fish (11, 16). These isolates were immunologically identical to each other but distinguishable from biotype 2 serovars using the same serotyping system (12). At least four additional serovars can be found using this serotyping system among biotype 1 isolates, although they have not been fully characterized (2; C. Amaro, unpublished data).

Due to the public health importance of this species and the difficulties in rapidly differentiating the strains with human virulence potential, several typing systems have been developed. The main genotyping systems are based on differences in the sequences of some loci, such as 16S rRNA, hemolysin (*vvhA*) genes, or the *vcg* (virulence-correlated gene) locus, which divide *V. vulnificus* populations in two genotypes, one primarily associated with environmental isolates and the other primarily associated with clinical isolates (7, 28, 33, 35). Most of the typing techniques described have been used for *V. vulnificus*, including randomly amplified polymorphic DNA analysis, repetitive extragenic palindromic PCR, and ribotyping, among others (5, 15, 23, 37, 39). These techniques are able to distinguish some specific groups within *V. vulnificus*; however, the majority of studies were performed with strain collections biased toward bio-

* Corresponding author. Mailing address: Departamento de Microbiología y Ecología, Universidad de Valencia, Avda. Dr. Moliner 50, 46100 Burjassot, Valencia, Spain. Phone: 34-96-3543104. Fax: 34-96-3544570. E-mail: carmen.amaro@uv.es.

‡ Present address: South Texas Center for Emerging Infectious Diseases and Department of Biology, The University of Texas at San Antonio, One UTSA Circle, San Antonio, TX 78249.

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TABLE 1. Strains used in this study and some of their properties

Strain	Origin	Country and year of isolation	Biotype	Multiplex[a]	DNA polymorphism[b]				Ribogroup (Rt)[c]
					vcg type	*vvhA* type	16S rRNA type	Profile	
CECT 4869	Diseased eel	Belgium, 1990	1	BT1/3	E	2	A	2	AI (1)
CG106	Oyster	Taiwan, 1993	1	BT1/3	C	1	B	1	AI (2)
CECT 5168	Human blood	United States	1	BT1/3	C	1	B	1	AI (3)
N87	Human blood	Japan, 1987	1	BT1/3	C	1	B	1	AI (3)
YJ106	Human blood	Taiwan	1	BT1/3	C	1	B	1	AI (3)
CECT 5167	Human blood	Japan	1	BT1/3	C	1	B	1	AI (4)
MLT 362	Oyster	United States	1	BT1/3	C	1	B	1	AI (5)
VV 425	Oyster	United States	1	BT1/3	E	1	A	Atypical	AI (6)
ATCC 33816	Human blood	United States	1	BT1/3	C	1	B	1	AI (7)
CG110	Seawater	Taiwan, 1993	1	BT1/3	C	1	B	1	AI (7)
CG118	Seawater	Taiwan, 1993	1	BT1/3	C	1	B	1	AI (8)
E4	Oyster	United States	1	BT1/3	C	1	B	1	AI (9)
CG111	Seawater	Taiwan, 1993	1	BT1/3	C	1	B	1	AI (10)
MLT 364	Oyster	United States	1	BT1/3	C	1	B	1	AI (11)
VV 1003	Oyster	United States	1	BT1/3	C	1	B	1	AI (11)
95-8-7	Diseased eel	Denmark, 1995	2	BT2-nonSerE	E	2	A	2	AI (11)
CS9133	Human blood	South Korea	1	BT1/3	C	1	B	1	AI (12)
CECT 4608	Eel farm water	Spain, 1990	1	BT1/3	C	1	B	1	AII (13)
KH03	Human blood	Japan, 2003	1	BT1/3	C	1	B	1	AII (14)
CECT 4862	Diseased eel	Japan, 1979	2	BT2-SerE	E	2	A	2	AII (14)
CECT 5164	Human blood	United States	1	BT1/3	C	2	B	Atypical	AII (15)
Riu-3	Seawater	Spain, 2003	1	BT1/3	E	2	A	2	AII (16)
Riu-1	Seawater	Spain, 2003	1	BT1/3	E	2	AB	Atypical	AII (16)
94385	Leg wound	Spain, 2001	1	BT1/3	E	2	B	Atypical	AII (17)
V4	Human blood	Australia	1	BT1/3	C	1	B	1	AII (18)
PD-2-52	Eel tank water	Spain, 2003	2	BT2-nonSerE	E	2	A	2	BI (19)
PD-2-58	Eel tank water	Spain, 2003	2	BT2-nonSerE	E	2	A	2	BI (20)
CECT 4917	Diseased eel	Spain, 1997	2	BT2-SerE	E	2	A	2	BI (21)
CECT 4998	Diseased eel	Spain, 1997	2	BT2-SerE	E	2	A	2	BI (21)
JE	Oyster	United States	1	BT1/3	E	2	B	Atypical	BI (21)
CECT 5165	Seawater	United States	1	BT1/3	E	2	A	2	BI (22)
A2	Diseased eel	Spain, 2000	1	BT1/3	E	2	A	2	BI (23)
An4	Diseased eel	Spain, 2000	1	BT1/3	E	2	A	2	BI (23)
An5	Diseased eel	Spain, 2000	1	BT1/3	E	2	A	2	BI (23)
An6	Diseased eel	Spain, 2000	1	BT1/3	E	2	A	2	BI (23)
CECT 4606	Eel tank water	Spain, 1990	1	BT1/3	E	2	A	2	BI (23)
PD-1	Eel tank water	Spain, 2001	1	BT1/3	E	2	A	2	BI (23)
PD-12	Eel tank water	Spain, 2001	1	BT1/3	E	2	A	2	BI (23)
PD-3	Eel tank water	Spain, 2001	1	BT1/3	E	2	A	2	BI (23)
PD-5	Eel tank water	Spain, 2001	1	BT1/3	E	2	A	2	BI (23)
V1	Eel tank water	Spain, 2001	1	BT1/3	E	2	A	2	BI (23)
CECT 4605	Diseased eel	Spain, 1990	2	BT2-SerE	E	2	A	2	BI (23)
11028	Human disease	Israel, 1996	3	BT1/3	E	1	AB	3	BI (24)
162	Human disease	Israel, 1997	3	BT1/3	E	1	AB	3	BI (24)
97	Human disease	Israel, 1997	3	BT1/3	E	1	AB	3	BI (24)
vv12	Human disease	Israel, 1996	3	BT1/3	E	1	AB	3	BI (24)
vv32	Human disease	Israel	3	BT1/3	E	1	AB	3	BI (24)
CECT 5169	Human blood	United States	1	BT1/3	C	1	B	1	BII (25)
94-9-119	Human disease	Denmark, 1994	1	BT1/3	E	2	A	2	BII (25)
CECT 4867	Unknown	Unknown	1	BT1/3	E	2	A	2	BII (25)
YN03	Human blood	Japan, 2003	1	BT1/3	E	2	A	2	BII (25)
535	Diseased eel	Sweden	2	BT2-nonSerE	E	2	A	2	BII (25)
536	Diseased eel	Sweden	2	BT2-nonSerE	E	2	A	2	BII (25)
960426-1/4C	Diseased eel	Denmark, 1996	2	BT2-nonSerE	E	2	A	2	BII (25)
960717-1/2F	Diseased eel	Denmark, 1996	2	BT2-nonSerE	E	2	A	2	BII (25)
A10	Diseased eel	Spain, 2002	2	BT2-nonSerE	E	2	A	2	BII (25)
A11	Diseased eel	Spain, 2002	2	BT2-nonSerE	E	2	A	2	BII (25)
A13	Diseased eel	Spain, 2002	2	BT2-nonSerE	E	2	A	2	BII (25)
A14	Diseased eel	Spain, 2002	2	BT2-nonSerE	E	2	A	2	BII (25)
CECT 5198	Diseased eel	Spain, 1999	2	BT2-nonSerE	E	2	A	2	BII (25)
CECT 5689	Diseased eel	Spain, 2002	2	BT2-nonSerE	E	2	A	2	BII (25)
CECT 5768	Diseased eel	Spain, 2001	2	BT2-nonSerE	E	2	A	2	BII (25)
CECT 5769	Diseased eel	Spain, 2002	2	BT2-nonSerE	E	2	A	2	BII (25)
90-2-11	Diseased eel	Denmark, 1990	2	BT2-SerE	E	2	A	2	BII (25)
94-8-112	Wound infection	Denmark, 1994	2	BT2-SerE	E	2	A	2	BII (25)
94-9-123	Seawater	Denmark, 1994	2	BT2-SerE	E	2	A	2	BII (25)
C1	Healthy eel	Spain, 2003	2	BT2-SerE	E	2	A	2	BII (25)
CECT 4602	Diseased eel	Spain, 1990	2	BT2-SerE	E	2	A	2	BII (25)
CECT 4603	Diseased eel	Spain, 1990	2	BT2-SerE	E	2	A	2	BII (25)
CECT 4604	Diseased eel	Spain, 1990	2	BT2-SerE	E	2	A	2	BII (25)
CECT 4864	Diseased eel	Spain, 1994	2	BT2-SerE	E	2	A	2	BII (25)
CECT 4868	Diseased eel	Norway, 1990	2	BT2-SerE	E	2	A	2	BII (25)
CECT 4870	Diseased eel	Sweden, 1991	2	BT2-SerE	E	2	A	2	BII (25)
CECT 5762	Healthy eel	Spain, 2002	2	BT2-SerE	E	2	A	2	BII (25)
CECT 898	Diseased eel	Japan, 1979	2	BT2-SerE	E	2	A	2	BII (25)
CIP 81.90	Human blood	France, 1981	2	BT2-SerE	E	2	A	2	BII (25)

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TABLE 1—*Continued*

Strain	Origin	Country and year of isolation	Biotype	Multiplex[a]	DNA polymorphism[b]				Ribogroup (Rt)[c]
					vcg type	*vvhA* type	16S rRNA type	Profile	
G83	Fish	South Korea	1	BT1/3	E	1	B	Atypical	BII (25)
VV 352	Seawater	United States	1	BT1/3	E	1	A	Atypical	BII (25)
MLT 406	Seawater	United States	1	BT1/3	E	2	A	2	BII (26)
95-8-6	Diseased eel	Denmark, 1995	2	BT2-nonSerE	E	2	A	2	BII (26)
CECT 529^T	Human blood	United States	1	BT1/3	E	1	A	Atypical	BII (26)
CECT 4174	Diseased eel	Japan, 1979	2	BT2-SerE	E	2	A	2	BII (27)
CG100	Oyster	Taiwan, 1993	1	BT1/3	C	1	B	1	BII (28)
L49	Brackish water	Japan	1	BT1/3	E	2	A	2	BII (29)
CECT 4607	Diseased eel	Spain, 1992	2	BT2-SerE	E	2	A	2	BII (30)
CECT 4999	Diseased eel	Spain, 1999	2	BT2-SerE	E	2	A	2	BII (30)
PD-2-66	Eel tank water	Spain, 2003	1	BT1/3	E	2	B	Atypical	BII (30)
CECT 4601	Diseased eel	Spain, 1989	2	BT2-SerE	E	2	A	2	BII (31)
94-9-130	Seawater	Denmark, 1994	1	BT1/3	E	2	A	2	BII (32)
CECT 7029	Diseased eel	Denmark, 2004	2	BT2-nonSerE	E	2	A	2	BII (32)
CECT 7030	Diseased eel	Denmark, 2004	2	BT2-nonSerE	E	2	A	2	BII (32)
95-8-162	Diseased eel	Denmark, 1995	2	BT2-nonSerE	E	2	A	2	BII (33)
CECT 4863	Leg wound	United States	2	BT2-SerE	E	2	A	2	BII (34)
CECT 897	Diseased eel	Japan, 1979	2	BT2-SerE	E	2	A	2	BII (35)
95-8-161	Diseased eel	Denmark, 1995	2	BT2-nonSerE	E	2	A	2	BII (36)
CECT 5343	Diseased eel	Spain, 2000	2	BT2-nonSerE	E	2	A	2	BII (36)
MLT404	Seawater	United States	1	BT1/3	E	2	A	2	BII (37)
CECT 4865	Diseased shrimp	Taiwan	2	BT2-SerE	E	2	A	2	BII (38)
CECT 5139	Diseased eel	Spain, 1998	2	BT2-SerE	E	2	A	2	BII (38)
CECT 4866	Human blood	Australia	2	BT2-SerE	E	2	A	2	BII (39)
UE516	Diseased Japanese eel	Taiwan	2	BT2-SerE	E	2	A	2	BII (40)
94-9-118	Human disease	Denmark, 1994	1	BT1/3	E	2	A	2	B (41)
534	Diseased eel	Sweden	1	BT1/3	E	2	A	2	B (42)
PD-2-47	Eel tank water	Spain, 2003	2	BT2-SerE	E	2	A	2	BIII (43)
PD-2-51	Eel tank water	Spain, 2003	2	BT2-SerE	E	2	A	2	BIII (43)
CECT 5763	Eel tank water	Spain, 2002	2	BT2-SerE	E	2	A	2	BIII (44)
PD-2-50	Eel tank water	Spain, 2003	2	BT2-SerE	E	2	A	2	BIII (45)
PD-2-55	Eel tank water	Spain, 2003	2	BT2-SerE	E	2	A	2	BIII (45)
Riu-2	Seawater	Spain, 2003	2	BT2-SerE	E	2	A	2	BIII (46)
PD-2-56	Eel tank water	Spain, 2003	2	BT2-SerE	E	2	A	2	BIII (47)
CECT 5166	Wound infection	United States	1	BT1/3	E	2	B	Atypical	ND[d]

[a] BT1/3, biotype 1 or 3; BT2-nonSerE, biotype 2 and not serovar E; BT2-SerE, biotype 2 serovar E.
[b] Results of the DNA polymorphism study (see Materials and Methods for details).
[c] See Fig. 2. Rt, ribopattern.
[d] ND, not determined.

type 1, since they included few or no biotype 2 and 3 strains, and toward North America, since the majority of the isolates were from that geographical region (14, 37).

The main objective of our study was to validate the usefulness of two typing methodologies (ribotyping and polymorphisms at selected loci) with a wide collection of strains of the different biotypes from different sources and geographic regions, whose biochemical diversity was also analyzed by different methodologies (API 20E, API 20NE, and BIOLOG).

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 111 *V. vulnificus* strains of different biotypes from different sources and regions were used in this study (Table 1). The strains were maintained both as lyophilized stocks at room temperature (25°C) and as frozen stocks at −80°C in marine broth (Difco) plus 20% (vol/vol) glycerol. Strains were grown in Luria-Bertani broth or on Luria-Bertani agar containing 1% (wt/vol) (total concentration) NaCl at 28°C for 24 h.

Phenotypic analysis. (i) Biotyping. The biotypes of the strains were confirmed by multiplex PCR (34). This method allows identification at the species level and at the same time discrimination between biotypes 1/3 and 2 and, within biotype 2, discrimination of serovar E, the zoonotic serovar. The biotype 3 strains used in this study were previously biotyped (11).

(ii) API 20E and API 20NE analysis. API 20E and API 20NE test kits (bioMerieux) were used according to the manufacturer's directions, with incubation of the strips at 28°C. Bacterial suspensions in saline solution or in AUX medium plus NaCl at a final concentration 1% (wt/vol) were used as the inocula for API 20E and API 20NE kits, respectively (10). Examination of the strips was conducted after 24 and 48 h. API profiles were compared using API DataBase version 4.0 for API 20E strips and version 6.0 for API 20NE strips (APILAB Software, version 3.3.3, Apilab Plus; bioMerieux).

(iii) BIOLOG analysis. BIOLOG-GN MicroPlates (BIOLOG) were used to evaluate substrate utilization patterns of the strains. The cells were streaked on BIOLOG Universal Growth agar (Oxoid) supplemented with 5% sheep erythrocytes (BUG-S) and incubated for 24 h at 28°C. Wells of a plate were inoculated with 150 μl of bacterial suspensions adjusted to the appropriate density in saline solution. The inoculated microplates were incubated at 28°C for 24 and 48 h and analyzed using a BIOLOG Microstation reader. Test results were obtained and identification (BIOLOG Microlog 6.01 database) was performed using BIOLOG MicroLog 3 software (BIOLOG), applying the automatic threshold option. Differences in the use of carbon substrates between the different groups were analyzed using the chi-square test function at $\alpha = 0.05$, employing SPSS 14.0 for Windows. Bionumerics software version 4.0 (Applied Maths) was used to cluster the strains based on their substrate utilization patterns using the unweighted-pair group method using arithmetic average (UPGMA) and two different similarity coefficients, the Jaccard and simple matching coefficients.

Genetic fingerprinting. (i) DNA sequence polymorphisms. The polymorphisms at selected loci were determined by PCR analysis of all *V. vulnificus* strains. Differentiation between the described alleles of the hemolysin gene (*vvhA*) and the 16S rRNA gene was performed under conditions described elsewhere (35). *vcg* typing for the environmental (type E) or clinical (type C) genotype was performed as described by Rosche et al. (33). In all assays, ca. 250 ng of DNA per 25 μl of reaction mixture was amplified using the high-fidelity Expand PCR system (Roche Diagnostics) in a TC-312 thermal cycler (Techne). The existence of an association between polymorphism and group (biotype, origin, or serovar) was calculated using the Pearson chi-square test function at $\alpha = 0.05$, employing SPSS 14.0 for Windows.

TABLE 2. Carbon sources used by *V. vulnificus* strains

Carbon source	Type	Growth[a]					
		Species (n = 111)	Biotype 1 (n = 51)	Biotype 2			Biotype 3 (n = 5)
				Total (n = 55)	Serovar E (n = 36)	Non-serovar E (n = 19)	
Dextrin	Polymer	+	+	+	+	+	+
Glycogen	Polymer	+	+	+	+	+	+
α-D-Glucose	Carbohydrate	+	+	+	+	+	+
D-Trehalose	Carbohydrate	+	+	+	+	+	+
N-Acetyl-D-glucosamine	Carbohydrate	+	+	+	+	+	+
D-Fructose	Carbohydrate	+	+	+	+	+	+
L-Asparagine	Amino acid	+	+	+	+	+	+
Glucose 6-phosphate	Phosphorylated chemical	+	+	+	+	(+)	+
Tween 80	Polymer	+	+	+	+	+	(+)
L-Glutamic acid	Amino acid	+	+	(+)	(+)	+	+
Inosine	Aromatic chemical	+	+	(+)	(+)	+	+
Maltose	Carbohydrate	(+)	(+)	+	+	(+)	+
D-Gluconic acid	Carboxylic acid	(+)	+	(+)	(+)	+	+
Cellobiose[b,c]	Carbohydrate	(+)	(+)	+	+	+	−
D-Mannose	Carbohydrate	(+)	(+)	(+)	(+)	(+)	(+)
Methylpyruvate	Ester	(+)	+	(+)	(+)	+	(+)
L-Aspartic acid	Amino acid	(+)	+	(+)	(+)	(+)	+
Gentiobiose[b,c]	Carbohydrate	(+)	(+)	+	+	(+)	−
Tween 40	Polymer	(+)	(+)	(+)	(+)	(+)	+
Succinic acid	Amide	(+)	(+)	(+)	(+)	(+)	+
Glucose 1-phosphate	Phosphorylated chemical	(+)	(+)	(+)	(+)	(+)	(+)
Monomethylsuccinate	Ester	(+)	(+)	V (73)	V (69)	(+)	+
L-Alanylglycine	Amino acid	V (74)	V (69)	(+)	(+)	(+)	V (60)
Glycyl-L-aspartic acid[c]	Amino acid	V (73)	V (59)	(+)	(+)	(+)	(+)
N-Acetyl-D-galactosamine	Carbohydrate	V (72)	V (67)	(+)	V (72)	(+)	(+)
Uridine[d]	Aromatic chemical	V (72)	(+)	V (69)	V (59)	(+)	(+)
L-Alanine	Amino acid	V (66)	V (63)	V (69)	V (72)	V (63)	V (60)
β-Methyl-D-glucoside[b,c]	Carbohydrate	V (59)	V (37)	(+)	(+)	(+)	−
DL-Lactic acid[b,c,d]	Carboxylic acid	V (59)	V (73)	V (44)	V (33)	V (63)	(+)
Glycerol	Alcohol	V (57)	V (59)	V (55)	V (56)	V (53)	V (60)
D-Galactose	Carbohydrate	V (54)	V (57)	V (55)	V (47)	V (68)	V (20)
Bromosuccinic acid	Brominated chemical	V (51)	V (53)	V (47)	V (44)	V (53)	(+)
DL-α-Glycerolphosphate[d]	Phosphorylated chemical	V (48)	V (49)	V (46)	V (56)	V (26)	V (60)
L-Proline	Amino acid	V (47)	V (43)	V (47)	V (47)	V (47)	(+)
Acetic acid	Carboxylic acid	V (38)	V (45)	V (35)	V (36)	V (32)	−
L-Threonine	Amino acid	V (38)	V (45)	V (35)	V (31)	V (42)	−
D-Mannitol[c,d]	Carbohydrate	V (36)	V (39)	V (36)	−	(+)	−
D-Psicose	Carbohydrate	V (35)	V (31)	V (38)	V (44)	V (26)	V (40)
L-Serine[b]	Amino acid	V (34)	V (43)	V (29)	V (28)	V (32)	−
D-Glucuronic acid[d]	Carboxylic acid	V (33)	V (28)	V (38)	V (28)	V (58)	V (40)
α-Ketoglutaric acid[d]	Carboxylic acid	V (32)	V (39)	V (27)	V (22)	V (37)	−
Glucuronamide	Amide	V (32)	V (29)	V (33)	V (25)	V (47)	V (40)
Glycyl-L-glutamic acid	Amino acid	V (32)	V (28)	V (35)	V (33)	V (37)	V (40)
α-D-Lactose[b,c]	Carbohydrate	V (31)	V (16)	V (47)	V (50)	V (43)	−
Thymidine[b,c]	Aromatic chemical	V (31)	V (41)	V (18)	−	V (32)	V (60)
Alaninamide	Amide	−	V (22)	V (22)	−	V (37)	−
ρ-Hydroxyphenylacetic acid	Carboxylic acid	−	V (25)	V (16)	V (22)	−	−
D-Alanine	Amino acid	−	VV (22)	−	−	V (16)	−
Propionic acid[b]	Carboxylic acid	−	−	V (20)	V (19)	V (21)	−

[a] +, positive reaction for ≥90% of the isolates; (+), positive reaction for 75 to 89% of the isolates; V, positive reaction for 11 to 74% of the isolates (the number in parentheses is the percentage of positive isolates); −, positive reaction for ≤10% of the isolates.

[b] Carbon source for which there are significant statistical differences ($P < 0.05$) in its use between the three biotypes.

[c] Carbon source for which there are significant statistical differences ($P < 0.05$) in its use between the four groups (biotype 1, biotype 2 serovar E, biotype 2 non-serovar E, and biotype 3).

[d] Carbon source for which there are significant statistical differences ($P < 0.05$) in its use between the serovar E isolates and the rest of the biotype 2 isolates.

(ii) Automated ribotyping. Ribotyping of the *V. vulnificus* isolates was carried out with the Riboprinter system (Qualicon Inc.). The assay was performed under conditions recommended by the manufacturer using HindIII (Roche) at 400 U μl^{-1}. Riboprinter patterns were partially processed by the Riboprinter system software in order to reduce background noise and to normalize the band positions using DNA size standards as references. The normalized patterns were then exported for further analysis as .txt files and imported into the Bionumerics software (version 4.0; Applied Maths) using LoadSamples script (DuPont Quali-

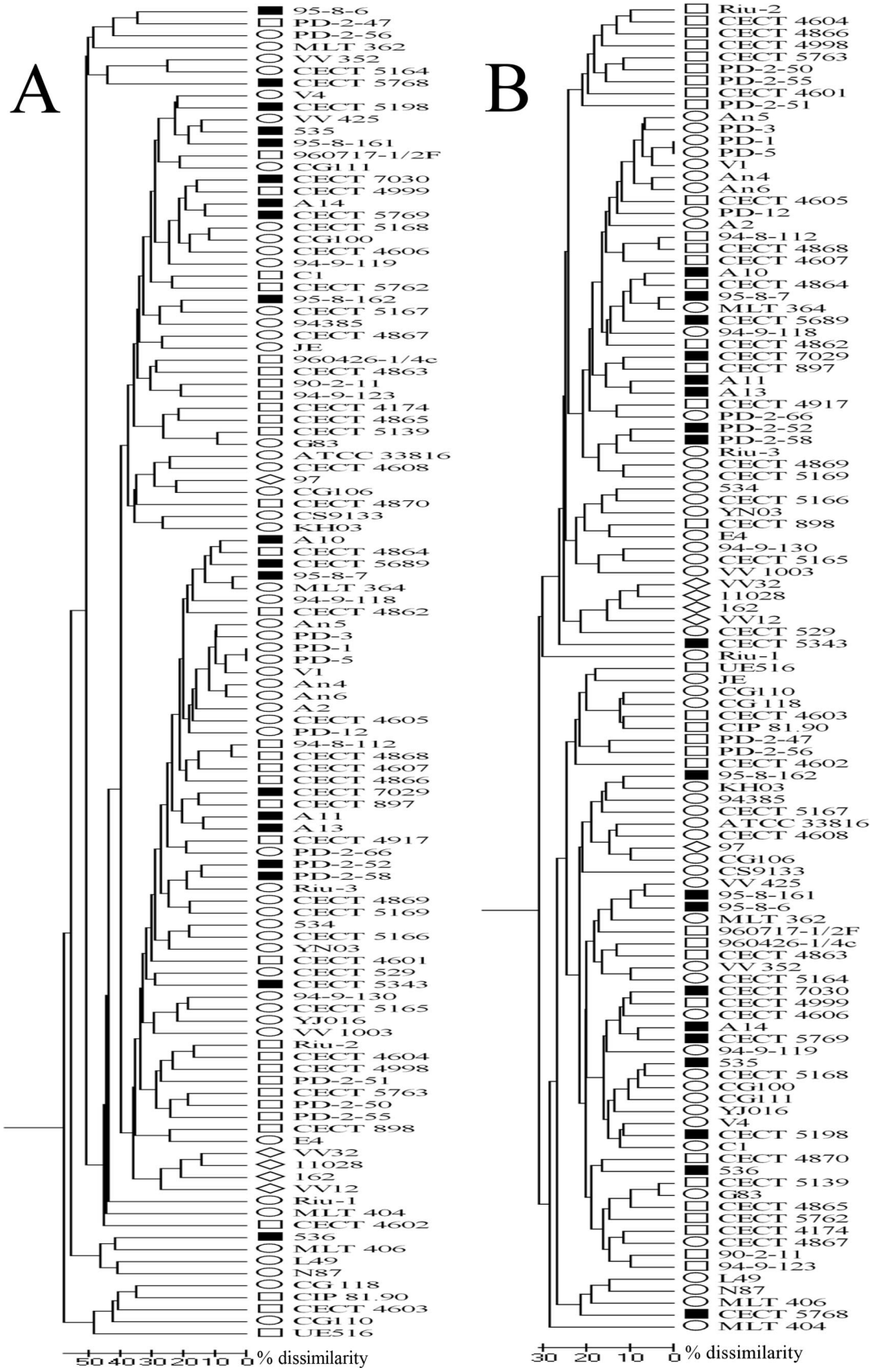

FIG. 1. Dendrograms based on UPGMA analysis of the BIOLOG results obtained for the *V. vulnificus* collection using the Jaccard (A) or simple matching (B) similarity coefficient. The scale bars indicate the percentage of dissimilarity. Biotype 1 strains are indicated by open ellipses, biotype 2 serovar E strains are indicated by open rectangles, biotype 2 non-serovar E strains are indicated by filled rectangles, and biotype 3 strains are indicated by open diamonds.

TABLE 3. Distribution of genotypes and genotypic profiles among *V. vulnificus* biotypes according to strain origin

Biotype	Origin	*n*	% with genotype[a]:							% with genotypic profile[b]:			
			vcg type C	*vcg* type E	*vvh* type 1	*vvh* type 2	rRNA type A	rRNA type B	rRNA type AB	1	2	3	Atypical
1	Water	19	21	79	26	74	68	26	5	21	63	0	16
	Oyster	8	75	25	87	13	13	87	0	75	0	0	25
	Fish	6	0	100	17	83	83	17	0	0	83	0	17
	Human blood	12	83	17	83	17	17	83	0	75	8	0	16
	Human (not blood)	4	0	100	0	100	50	50	0	0	50	0	50
2	Water	10	0	100	0	100	100	0	0	0	100	0	0
	Healthy fish	2	0	100	0	100	100	0	0	0	100	0	0
	Diseased fish	38	0	100	0	100	100	0	0	0	100	0	0
	Diseased shrimp	1	0	100	0	100	100	0	0	0	100	0	0
	Human blood	2	0	100	0	100	100	0	0	0	100	0	0
	Human (not blood)	2	0	100	0	100	100	0	0	0	100	0	0
3	Bacteremia	5	0	100	100	0	0	0	100	0	0	100	0

[a] Percentage of isolates with positive PCR results.
[b] Percentage of isolates with each profile. See the text for a description of the profiles.

con). Clustering analysis was performed by UPGMA based on the Dice coefficient for band matching, with a position tolerance and an optimization setting of 1%. Bands for band matching were assigned automatically and manually edited if necessary.

RESULTS

Phenotypic analysis. (i) API 20E and API 20NE analysis. A total of 25 different API 20E and API 20NE profiles were obtained for the *V. vulnificus* collection (see Tables S1 and S2 in the supplemental material). In the case of the API 20E system, only 60% of the strains were correctly identified as *V. vulnificus*, and the percentage was 20% for biotype 3 isolates. The remaining strains gave a mixed profile or were misidentified as *Burkholderia cepacia* or *Vibrio parahaemolyticus*. Three main profiles were detected for biotype 1 isolates (5346105 [19.6%], 5146105 [15.7%], and 5346005 [17.6%]), two main profiles were detected for biotype 2 serovar E isolates (5006005 [29.4%] and 5206005 [23.5%]), two main profiles were detected for biotype 2 non-serovar E isolates (5146105 [38%] and 5346105 [33%]), and one main profile was detected for biotype 3 isolates (4146004 [40%]) (see Table S1 in the supplemental material). None of the strains was correctly identified as *V. vulnificus* with the API 20NE system. Instead, most of the isolates were identified at the genus level as *Aeromonas* or *Vibrio*, and the species *Aeromonas hydrophila* and *Vibrio cholerae* were the most frequent options (see Table S2 in the supplemental material). The main profile exhibited by biotype 1 isolates was 7476745 (43.1%), the main profile exhibited biotype 2 serovar E isolates was 5472745 (73.6%), the main profile exhibited by biotype 2 non-serovar E isolates was 7476745 (61.9%), and the main profile exhibited by biotype 3 strains was 7062745 (40%).

(ii) BIOLOG GN2 plates. The BIOLOG system correctly identified 84% of the 111 isolates studied. Eight of the low-discrimination identifications (7% of the strains) were listed as *V. vulnificus* as the first option, although with low probability. The carbon sources that *V. vulnificus* was able to oxidize are shown in Table 2. On average, 32 carbon substrates were utilized. *V. vulnificus* strains did not use amines or carboxylic acids, with the exception of D-glucoronic and succinic acid, which were metabolized by more than the 80% of the strains examined (Table 2). There was no specific profile (expressed as the carbon sources utilized by an isolate) that could be assigned to a particular group of strains. In fact, only two strains, isolated from the same water sample (PD-1 and PD-5), used the same carbon sources. In addition, cluster analysis performed by applying either the Jaccard index, which takes into account the similarity based on the number of positive coincidences, or simple matching, which takes into account the positive and negative coincidences, revealed that most of the groups of strains were not related to a common biotype, serovar, or origin (Fig. 1). Such variability in the use of carbon sources caused difficulties in attempts to establish a reliable test that could be used for discriminating biotypes or serovars. Nevertheless, we found statistical differences for the differential use of some carbon sources between groups (Table 2). Some examples are the use of the cellobiose and gentibiose, which was negative for the biotype 3 isolates but positive for the rest of the strains tested. The biotype 2 strains were able to oxidize β-methyl-D-glucoside; however, no biotype 3 and few biotype 1 isolates were able to use this carbon source.

Genetic diversity observed with DNA polymorphism locus typing. Results of the multiplex PCR (34) analysis are shown in Table 1. The allelic distribution among environmental, human, and fish *V. vulnificus* isolates for the three biotypes is shown in Table 3. Biotype 1 strains from oysters and human blood predominantly were *vcg* type C, whereas the biotype 1 strains from fish and nonsepticemic human infections and most of the biotype 1 isolates from water, together with biotype 2 and 3 isolates regardless of their origin, were *vcg* type E. We detected *vvhA* gene type 1 in biotype 1 strains from oysters and human septicemia together with biotype 3 strains from human bacteremia, whereas biotype 1 strains from fish and human wounds and all biotype 2 strains, irrespective of their origin, were *vvhA* type 2. In contrast to the *vcg* results, we observed more variability in the *vvhA* typing results with the water isolates of biotype 1. These isolates, together with those from human wounds, also showed variable results for the 16S rRNA gene

polymorphisms, while fish biotype 1 and biotype 2 isolates, regardless of their origin, were type A. Oyster and human blood biotype 1 isolates were type B, and biotype 3 isolates were type AB. Thus, three main genotypic profiles were found among the collection of *V. vulnificus* isolates. Profile 1 consisted of genotype *vcg* type C, 16S rRNA type B, and *vvh* type 1 and was exhibited by biotype 1 strains from human septicemia and oysters. Profile 2 consisted of genotype *vcg* type E, 16S rRNA type A, and *vvh* type 2 and was exhibited by biotype 2 isolates, regardless of their origin, and by biotype 1 isolates from fish and water and some human isolates. Profile 3 consisted of genotype *vcg* type E, 16S rRNA type AB, and *vvh* type 2 and was exhibited only by biotype 3 strains. No specific profile was found for water and human nonblood isolates of biotype 1, which showed variable results. Some atypical profiles were also found, such as the profile showed by environmental biotype 1 isolate Riu-1, which was positive for both types of 16S rRNA (as were the biotype 3 strains) and possessed hemolysin type 2.

Automated ribotyping. Figure 2 shows the dendrogram obtained from the normalized ribotypes (Rt) after UPGMA clustering. The Riboprinter generated some bands with low intensity, especially above 15 kb, that probably corresponded to undigested DNA as they were not reproducible when selected strains were ribotyped a second time. These bands, together with those at <1 kb, were not taken into account in the ribotyping cluster analysis. Forty-seven Rt were distinguished among the 111 *V. vulnificus* strains (Table 1), which grouped at a similarity level of 62%. We included two strains of other *Vibrio* species as outgroups, *V. harveyi* and *V. parahaemolyticus*, which were clearly different from the strains of *V. vulnificus* and which grouped at 45% similarity (Fig. 2). Two main groups of *V. vulnificus* strains could be distinguished. Division A included 92% of the biotype 1 strains and the majority of human blood (70%) and oyster (75%) isolates with profile 1, whereas division B included some biotype 1 strains from human wounds, fish, and water with profile 2 and the majority of biotype 2 (96%) and 3 (100%) isolates with profiles 2 and 3, respectively. These two divisions could be subdivided into five groups (ribogroups AI, AII, BI, BII, and BIII) based on ≥80% similarity, even though five strains (CECT 4869, CG106, V4, 94-9-118, and 534) did not cluster with other strains (Fig. 2). All of the strains with profile 1 were clustered in ribogroups AI and AII, except for a strain from human septicemia (CECT 5169) and an environmental strain (CG100). Ribogroups AI, AII, and BIII included strains with a unique biotype; ribogroups AI and AII included biotype 1 strains, mostly from humans, and ribogroup BIII included biotype 2 and serovar E strains isolated from an unusual source, brackish water of the estuary of the River Ebro (Mediterranean Sea). The rest of the biotype 2 strains were included in ribogroup BII, and most of them exhibited the same Rt pattern (Rt 25) regardless of the serovar, while all the strains of biotype 3 were grouped in ribogroup BI, which also showed a unique Rt (Rt 24).

DISCUSSION

In order to validate the genotyping systems designed for differentiating clinical from environmental *V. vulnificus* isolates, a selection of these systems was used with a large collection of strains of different biotypes and serovars recovered from sources worldwide. The phenotypic diversity of the collection was analyzed first with three miniaturized bacterial identification systems, whose usefulness for identification of *V. vulnificus* at the specific or intraspecific level was evaluated in parallel. The BIOLOG system was the most effective system, giving 84% correctly identified strains. The BIOLOG results showed that *V. vulnificus* has the ability to oxidize a great variety of carbon sources. This species is also highly heterogeneous, since almost every isolate had a unique profile. The percentage of correctly identified strains was 60% when the API 20E system was used and 0% with the API 20NE system. These results are in agreement with previous reports on the doubtful usefulness of both API systems for identification of clinical and environmental *V. vulnificus* isolates (10, 17, 18, 29), although they are still being used, mostly for clinical diagnosis. Based on these results, the BIOLOG system is the most adequate system for *V. vulnificus* identification at the species level, and the other two systems, especially the API 20NE system, should not be used unless the databases are updated with the profiles found in the present work. When the utility of the three systems for intraspecific classification was considered, none of them was able to distinguish biotype 2 non-serovar E (serovar A/I) isolates from biotype 1 isolates, although several API 20E and API 20NE profiles were found to be specific for biotype 2 serovar E and biotype 3 strains (see Tables S1 and S2 in the supplemental material). Inclusion of these profiles in the API database would facilitate correct identification of more *V. vulnificus* isolates and in some cases subclassification into biotype 2 serovar E or biotype 3. In general, however, the profiles would not allow discrimination of other biotypes or groups. Despite finding several tests in the BIOLOG system that revealed significant differences between groups, we found that using these tests was not adequate for good discrimination. Only the combination of negative results for cellobiose and gentibiose breakdown allowed allocation of isolates to biotype 3.

Previous studies have proposed various genetic methods to distinguish strains of this species with human-pathogenic potential (14, 28, 33, 35). We performed an analysis of three of these methods, the 16S rRNA, *vvhA*, and *vcg* methods, with our *V. vulnificus* collection. According to previous reports, most *V. vulnificus* human isolates should be *vcg* type C, *vvhA* type 1, and 16S rRNA type B (genotypic profile 1 in our study), and most environmental isolates should be *vcg* type E, *vvhA* type 2, and 16S rRNA type A (genotypic profile 2 in our study). However, we were able to establish an association only between genotypic profile 1 and human isolates for biotype 1 strains from septicemic cases, regardless of their geographical origin. The remaining biotype 1 human isolates mostly exhibited genotype profile 2, like the majority of the environmental biotype 1 isolates, except those from oysters. Our results for oyster isolates are opposite the results obtained by other workers (22, 28, 33) and could be due to the inclusion of isolates from Asia, where a major proportion of 16S rRNA type B has been reported (25). In previous studies variation between ratios of 16S rRNA type A to rRNA type B were observed for different sampling points or water temperatures (22, 27, 39). For the rest of the isolates, we found an association between profile and biotype rather than an association between profile

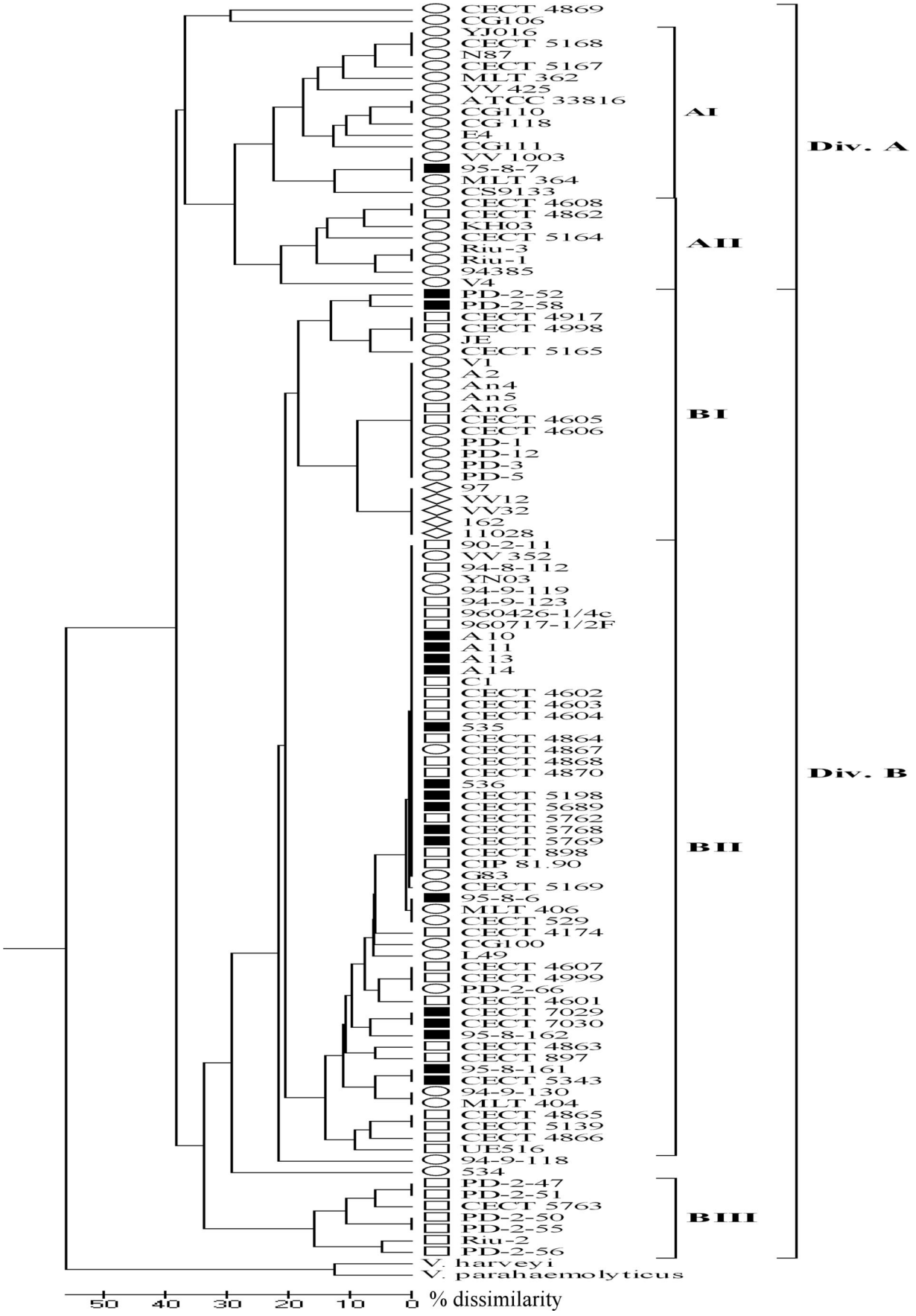

FIG. 2. Dendrogram based on UPGMA cluster analysis of the ribotypes obtained in this study. The scale bar indicates the percentage of dissimilarity. Divisions A and B and the five ribogroups are shown. The symbols are the same as those used in Fig. 1.

and origin of the isolate. Thus, all biotype 2 strains, regardless of their source (human, fish, or water), exhibited profile 2, and all biotype 3 strains, all from human bacteremia, exhibited profile 3. The variability in profiles observed among biotype 1 strains can be attributed to the greater genetic variability of this biotype. Genetic characterization of our *V. vulnificus* collection performed by ribotyping confirmed this observation. Ribotyping is a general technique also used in epidemiological studies of *V. vulnificus* (3, 5, 6, 9, 19, 23). We selected HindIII to perform the DNA digestion since it has been reported to provide the best discrimination between biotypes (9, 18). A common pattern was observed for all *V. vulnificus* strains, and a group of bands between 2 and 3 kb was absent in the profiles of the other vibrios examined. These bands were also observed in previous studies with manual protocols (9, 18, 22). The strains were grouped on the basis of their similarities in ribopatterns into two divisions and five groups. As expected, biotype 1 strains were found in almost every group and subgroup. Nevertheless, the biotype 1 strains that exhibited profile 1 were located mostly in division A, while the second division included all the biotype 2 and 3 strains together with additional biotype 1 strains from environmental sources and wound infections. In this division, the major genotypic profiles were profiles 2 and 3. Interestingly, ribogroup BI was comprised of all biotype 3 isolates that were closely related to environmental biotype 1 strains from sites related to fish farms. This result supports the previous hypothesis concerning the origin of biotype 3 as a clone associated with tilapia culture in Israel that recently emerged (13).

The eel-pathogenic strains were located in division B, with a major Rt (Rt 25) that included strains of the different serovars as well as other biotype 1 strains isolated mainly from the environment or from human wounds. This result also supports the hypothesis that biotype 2 strains could have been emerged from biotype 1 strains present in the environment. Recently, it has been shown that eel virulence relies on a 68-kb plasmid that can be transmitted between strains by conjugation with the aid of a conjugative plasmid (26). In this scenario, biotype 2 strains could have evolved independently by acquisition of the virulence plasmid by different clones of biotype 1 strains in the environment. Only one of these clones (serovar E) could be phenotypically distinguished from the rest of the biotypes, while the rest (serovars A and I) could not be distinguished. Additional studies based on multilocus sequence analysis with biotype 2 strains of different serovars and from different sources are needed to confirm this hypothesis.

Ribotyping has been used for differentiating clinical and environmental *V. vulnificus* isolates and biotypes, and several correlations between ribopatterns and geographic origin have been found (5, 6, 8). Our results, however, suggest that this technique may be useful for revealing genetic relationships among *V. vulnificus* isolates, but it is not likely to be useful for rapid identification of strains with public health interest.

In conclusion, the results obtained in the present work demonstrate that the species *V. vulnificus* is highly heterogeneous and that most of the diversity is present in biotype 1. Biotype 2 and 3 strains, in contrast, are more homogeneous, even though biotype 2 is serologically and phenotypically heterogeneous. There is a need for methods capable of rapid, sensitive, and accurate identification of the strains dangerous for public health. The DNA polymorphisms studied have been proposed for routine monitoring of the quality of seafood and water, but our results suggest that their use could eliminate samples containing strains with human-pathogenic potential, such as biotype 2 serovar E and biotype 3 strains. It is clear, therefore, that new genetic markers with epidemiological potential need to be found to clearly differentiate *V. vulnificus*.

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