Applied and Environmental Microbiology, Jan. 2011, p. 688–695 Vol. 77, No. 2
0099-2240/11/$12.00 doi:10.1128/AEM.01263-10

Polyphyletic Origin of *Vibrio vulnificus* Biotype 2 as Revealed by Sequence-Based Analysis▽†

Eva Sanjuán,[1] Fernando González-Candelas,[2,3] and Carmen Amaro[1]*

Departamento de Microbiología, Facultad de Biología, Universidad de Valencia, Valencia, Spain[1]; Unidad Mixta Genómica y Salud Centro Superior de Investigación en Salud Pública—Universidad de Valencia/Instituto Cavanilles de Biodiversidad y Biología Evolutiva, Valencia, Spain[2]; and CIBER de Epidemiología y Salud Pública, Valencia, Spain[3]

Received 28 May 2010/Accepted 8 November 2010

A sequence-based analysis of seven housekeeping and virulence-related genes shows that the species *Vibrio vulnificus* is subdivided into three phylogenetic lineages that do not correspond with the biotypes and that biotype 2 is polyphyletic. These results support the reclassification of biotype 2 as a pathovar that would group the strains with pathogenic potential to develop vibriosis in fish.

Vibrio vulnificus is a pathogenic bacterial species that inhabits brackish waters in warm and tropical ecosystems (9, 11, 24). The species is highly heterogeneous and has been subdivided into three biotypes. Biotype 1 is the most abundant; it is distributed worldwide and causes sporadic cases of human vibriosis. Biotype 2 is also distributed worldwide, and it is the only one that harbors the genetic information to infect both fish and humans. Finally, biotype 3 is geographically restricted to Israel, and it causes outbreaks of human vibriosis after fish handling (1, 4, 13, 25). Biotype 2 is further subdivided into serovars A, E, and I (7; unpublished results), serovar E being the one associated with human vibriosis (1).

The genes essential for fish vibriosis are located in a recently described virulence plasmid that can be transmitted between strains cointegrated with a conjugative plasmid (12) that is present in almost 90% of biotype 2 isolates (17). In contrast, the genetic basis of human infection is poorly understood, since the putative virulence factors identified so far are found in both clinical and environmental isolates of the three biotypes (24, 27).

Several studies based on multilocus sequence typing (MLST) of housekeeping genes (2, 3, 6) and on ribotyping (21) suggest that the species is subdivided into two main evolutionary lineages with apparently different human pathogenic potential; one includes a majority of the human clinical isolates of biotype 1 (clinical branch), and the other a majority of the environmental isolates of the same biotype (environmental branch). The few isolates of biotype 2 studied are in the environmental branch, while biotype 3 strains are in a variable position depending on the study (2, 3, 6).

Given this scenario, the aim of this work has been to analyze the evolutionary origins of biotype 2, starting from the hypothesis that horizontal transfer of the virulence plasmid together with recombination events could have played a major role in the emergence of this biotype. To this end, a sequence-based analysis of three virulence-associated (*vvhA*, *wzz*, and *pilF*) and four housekeeping (*glp*, *mdh*, *pyrC*, and *pntA*) genes (the last ones selected from the MLST scheme for *V. vulnificus* [3]) was applied to a collection of 115 isolates that included strains of the three biotypes from clinical (humans and fish) and environmental sources (Table 1). The primer pairs for the genes are listed in Table S1 in the supplemental material. The genetic variability (θ) at the locus and biotype level was examined by using DnaSP4.09 (20). *pilF* and *wzz* (genes involved in surface antigen biogenesis) showed the highest levels of genetic variability (Table 2). Regarding the biotypes, biotype 1 showed the highest genetic variability, while biotype 2 was highly homogeneous, and no genetic diversity was observed among the biotype 3 isolates (see Table S2 in the supplemental material).

To analyze the phylogeny of the *V. vulnificus* collection, we constructed a maximum likelihood (ML) tree from the 3,159-bp concatenated sequence of the seven loci (Fig. 1) by using PHYML 2.4.4 (8). The most appropriate model for nucleotide substitution was assessed with Modeltest version 3.7 (16). The concatenated tree shows the isolates clustered into three main lineages (Fig. 1). Lineage I (LI) contained isolates of biotypes 1 and 2 from fish farms and isolates from diseased fish and humans infected through fish handling or water contact. This lineage is enriched in European isolates, probably because the fish-farming industry is especially developed in Europe, whose countries apply specific-pathogen-control programs. LII was formed by biotype 3 strains from Israel, and LIII included biotype 1 isolates mostly recovered from environmental samples or from human septicemic cases registered in the United States and Asia. The nucleotide diversity within each lineage was then examined, and it was found that LI and LIII have similar values (π in Table S2 in the supplemental material). The human isolates are genetically more diverse than those from environmental origins, and both are much more diverse than isolates from diseased animals. This result would suggest that multiple environmental clones have the ability to infect humans, which correlates with human cases being presented as sporadic infections worldwide, and that only a few clones are able to infect fish, although they are overrepresented by clone

* Corresponding author. Mailing address: Departamento de Microbiología, Facultad de Biología, Universidad de Valencia, 46100 Valencia, Spain. Phone: 34 96 354 31 04. Fax: 34 96 354 45 70. E-mail: carmen.amaro@uv.es.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 19 November 2010.

TABLE 1. Characteristics of the *V. vulnificus* isolates used in this study

Strain	Country and yr of isolation	Source	Biotype[a] and serovar	ST[b]	No. of allelic sequences[b] of:						
					glp	*pntA*	*pyrC*	*pilF*	*wzz*	*vvhA*	*mdh*
YJ106	Taiwan	Human blood	BT1/3	1	1	1	30	18	1	21	5
CECT 4608	Spain, 1990	Eel farm water	BT1/3	2	1	2	12	11	11	2	13
BT3-1033	Israel, 1996	Diseased human	BT1/3	3	2	5	2	10	13	5	10
BT3-11028	Israel, 1996	Diseased human	BT1/3	3	2	5	2	10	13	5	10
BT3-12	Israel, 1996	Diseased human	BT1/3	3	2	5	2	10	13	5	10
BT3-162	Israel, 1997	Diseased human	BT1/3	3	2	5	2	10	13	5	10
BT3-32	Israel	Diseased human	BT1/3	3	2	5	2	10	13	5	10
BT3-97	Israel, 1997	Diseased human	BT1/3	3	2	5	2	10	13	5	10
A13	Spain, 2002	Diseased eel	BT2 non-serovar E (serovar A)	4	3	7	3	1	2	7	6
CECT 5198	Spain, 1999	Diseased eel	BT2 non-serovar E (serovar A)	5	3	7	3	1	2	7	8
CECT 7029	Denmark, 2004	Diseased eel	BT2 non-serovar E (serovar A)	5	3	7	3	1	2	7	8
CECT 7030	Denmark, 2004	Diseased eel	BT2 non-serovar E (serovar A)	5	3	7	3	1	2	7	8
A10	Spain, 2002	Diseased eel	BT2 non-serovar E (serovar A)	5	3	7	3	1	2	7	8
CECT 5343	Spain, 2000	Diseased eel	BT2 non-serovar E (serovar A)	6	3	7	3	1	2	7	28
A11	Spain, 2002	Diseased eel	BT2 non-serovar E (serovar A)	7	3	7	3	1	2	7	32
CECT 5689	Spain, 2002	Diseased eel	BT2 non-serovar E (serovar A)	8	3	7	17	1	2	7	29
CECT 5768	Spain, 2001	Diseased eel	BT2 non-serovar E (serovar A)	9	3	7	17	1	2	7	30
CECT 5769	Spain, 2002	Diseased eel	BT2 non-serovar E (serovar A)	10	3	7	17	1	2	7	31
L49	Japan	Brackish water	BT1/3	11	3	7	26	1	2	7	6
PD-2-66	Spain, 2003	Eel tank water	BT1/3	12	3	8	26	25	28	15	8
JE	USA	Oyster	BT1/3	13	3	15	13	2	24	1	25
CECT 5166	USA	Human blood	BT1/3	14	3	18	15	14	16	2	14
CECT 4867	Unknown	Diseased eel	BT1/3	15	4	22	31	26	3	15	33
534	Sweden	Diseased eel	BT1/3	16	5	8	4	2	4	12	8
CECT 4869	Belgium, 1990	Diseased eel	BT1/3	17	5	8	13	2	14	19	1
A2	Spain, 2000	Eel tank water	BT1/3	18	6	11	4	2	11	2	7
An4	Spain, 2000	Eel tank water	BT1/3	18	6	11	4	2	11	2	7
An5	Spain, 2000	Eel tank water	BT1/3	18	6	11	4	2	11	2	7
An6	Spain, 2000	Eel tank water	BT1/3	18	6	11	4	2	11	2	7
An7	Spain, 2000	Eel tank water	BT1/3	18	6	11	4	2	11	2	7
PD-1	Spain, 2001	Eel tank water	BT1/3	18	6	11	4	2	11	2	7
PD-3	Spain, 2001	Eel tank water	BT1/3	18	6	11	4	2	11	2	7
PD-5	Spain, 2001	Eel tank water	BT1/3	18	6	11	4	2	11	2	7
V1	Spain, 2001	Eel tank water	BT1/3	18	6	11	4	2	11	2	7
PD-12	Spain, 2001	Eel tank water	BT1/3	19	6	11	4	23	11	2	7
CECT 4606	Spain, 1990	Healthy eel	BT1/3	20	6	11	4	4	3	2	8
MLT 362	USA	Environmental	BT1/3	21	7	4	21	8	25	16	33
VV 1003	USA	Environmental	BT1/3	22	8	23	28	27	29	14	24
MLT 364	USA	Environmental	BT1/3	23	9	24	24	8	9	16	8
MLT 406	USA	Environmental	BT1/3	24	10	19	26	2	3	2	1
MLT404	USA	Environmental	BT1/3	25	11	20	25	21	26	7	33
ATCC 33816	USA	Human blood	BT1/3	26	12	9	12	16	12	7	4
CG100	Taiwan, 1993	Oyster	BT1/3	27	12	9	12	15	20	9	2
V2	Unkown	Environmental	BT1/3	28	12	9	12	16	12	13	4
CG110	Taiwan, 1993	Seawater	BT1/3	29	12	9	19	15	12	4	D3
VV 352	USA	Environmental	BT1/3	30	12	9	29	16	12	17	19
CG111	Taiwan, 1993	Seawater	BT1/3	31	12	14	20	15	12	4	2
CG118	Taiwan, 1993	Seawater	BT1/3	32	12	14	20	15	12	4	4
CG106	Taiwan, 1993	Oyster	BT1/3	33	12	14	21	17	21	18	3
CECT 5167	Japan	Human blood	BT1/3	34	13	14	16	15	17	4	12
VV 425	USA	Environmental	BT1/3	35	13	21	4	18	31	5	27
CECT 5164	USA	Human blood	BT1/3	36	14	3	12	13	15	20	20
CECT 5169	USA	Human blood	BT1/3	37	15	7	4	17	19	11	22
CS9133	South Korea	Human blood	BT1/3	38	15	14	22	18	22	10	22
KH03	Japan, 2003	Human blood	BT1/3	39	16	14	12	19	18	4	23

Continued on following page

TABLE 1—*Continued*

Strain	Country and yr of isolation	Source	Biotype[a] and serovar	ST[b]	No. of allelic sequences[b] of:						
					glp	*pntA*	*pyrC*	*pilF*	*wzz*	*vvhA*	*mdh*
E4	USA	Seafood	BT1/3	40	16	14	21	19	18	4	26
V4	Australia	Human blood	BT1/3	41	17	14	27	18	30	22	23
94-9-118	Denmark, 1994	Expectoration from lungs	BT1/3	42	18	22	5	2	5	1	8
G83	South Korea	Fish	BT1/3	43	19	16	23	20	23	8	8
CECT 5168	USA	Human blood	BT1/3	44	20	7	4	16	18	4	21
YN03	Japan, 2003	Human blood	BT1/3	45	21	13	9	12	32	7	7
94385	Spain, 2001	Leg wound	BT1/3	46	22	14	8	6	8	8	7
536	Sweden	Diseased eel	BT2 non-serovar E	47	23	10	3	3	2	7	8
535	Sweden	Diseased eel	BT2 non-serovar E	48	23	10	3	3	2	7	15
CECT 5165	USA	Seawater	BT1/3	49	23	21	14	14	3	2	8
Riu-1	Spain, 2003	Seawater	BT1/3	50	24	16	8	20	7	8	7
94-9-130	Denmark, 1994	Water	BT1/3	51	25	8	7	5	7	1	8
Riu-3	Spain, 2003	Seawater	BT1/3	52	25	12	3	24	12	1	8
PD-2-58	Spain, 2003	Eel tank water	BT2 non-serovar E	53	25	12	3	24	3	1	8
PD-2-52	Spain, 2003	Eel tank water	BT2 non-serovar E	54	25	12	3	24	27	1	8
94-9-119	Denmark, 1994	Wound infection	BT1/3	55	26	17	6	2	6	1	8
A14	Spain, 2002	Diseased eel	BT2 non-serovar E	56	27	7	3	1	2	7	8
95-8-161	Denmark, 1995	Diseased eel	BT2 non-serovar E (serovar I)	57	28	7	9	7	2	7	16
95-8-162	Denmark, 1995	Diseased eel	BT2 non-serovar E (serovar I)	58	28	7	9	7	2	7	17
95-8-6	Denmark, 1995	Diseased eel	BT2 non-serovar E (serovar I)	59	28	7	10	7	2	7	8
95-8-7	Denmark, 1995	Diseased eel	BT2 non-serovar E (serovar I)	60	28	7	11	8	2	7	8
CIP 81.90	France	Human blood	BT2 serovar E	61	29	7	4	4	3	3	8
CECT 4862	Japan, 1979	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
90-2-11	Denmark, 1990	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
94-8-112	Denmark, 1994	Wound infection	BT2 serovar E	62	29	8	4	4	3	3	8
94-9-123	Denmark, 1994	Seawater	BT2 serovar E	62	29	8	4	4	3	3	8
CCUG 38521	Sweden, 1997	Wound infection	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4174	Japan, 1979	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4601	Spain, 1989	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4602	Spain, 1990	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4603	Spain, 1990	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4605	Spain, 1990	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4607	Spain, 1992	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4863	USA	Leg wound infection	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4864	Spain, 1994	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4865	Taiwan	Diseased shrimp	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4866	Australia	Human blood	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4870	Sweden, 1991	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4917	Spain, 1997	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 897	Japan, 1979	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 898	Japan, 1979	Diseased eel	BT2 serovar E	62	29	8	4	4	3	3	8
PD-2-47	Spain, 2003	Eel tank water	BT2 serovar E	62	29	8	4	4	3	3	8
PD-2-55	Spain, 2003	Eel tank water	BT2 serovar E	62	29	8	4	4	3	3	8
PD-2-56	Spain, 2003	Eel tank water	BT2 serovar E	62	29	8	4	4	3	3	8
Riu-2	Spain, 2003	Seawater	BT2 serovar E	62	29	8	4	4	3	3	8
UE516	Taiwan	Diseased Japanese eel	BT2 serovar E	62	29	8	4	4	3	3	8
CECT 4868	Norway, 1990	Diseased eel	BT2 serovar E	63	29	8	4	4	3	3	10
CECT 4999	Spain, 1999	Diseased eel	BT2 serovar E	64	29	8	4	4	3	3	16
C1	Spain, 2003	Healthy eel	BT2 serovar E	65	29	8	4	4	3	3	17
CECT 4604	Spain, 1990	Diseased eel	BT2 serovar E	66	29	8	4	4	3	3	18
CECT 4998	Spain, 1997	Diseased eel	BT2 serovar E	66	29	8	4	4	3	3	18
CECT 5139	Spain, 1998	Diseased eel	BT2 serovar E	66	29	8	4	4	3	3	18
PD-2-50	Spain, 2003	Eel tank water	BT2 serovar E	67	29	8	4	4	3	3	32
PD-2-51	Spain, 2003	Eel tank water	BT2 serovar E	67	29	8	4	4	3	3	32
CECT 5762	Spain, 2002	Healthy eel	BT2 serovar E	68	29	8	18	4	3	3	17
CECT 5763	Spain, 2002	Eel tank water	BT2 serovar E	69	29	8	18	4	3	3	8
CECT 529^T	USA	Human blood	BT1/3	70	29	18	15	14	16	2	14
960717-1/2F	Denmark, 1996	Diseased eel	BT2 non-serovar E	71	30	6	4	9	6	1	11
960426-1/4C	Denmark, 1996	Diseased eel	BT2 non-serovar E	72	30	6	4	9	10	6	11
N87	Japan, 1987	Human blood	BT1/3	73	31	4	1	22	33	4	9

[a] BT, biotype.

[b] Sequences from each locus were aligned using Vector NTI 9.0.0 software (Infomax) and were edited manually by visual inspection. Different allelic sequences within a locus were assigned arbitrary numbers. Each isolate was consequently given a 7-number sequence designated a sequence type (ST).

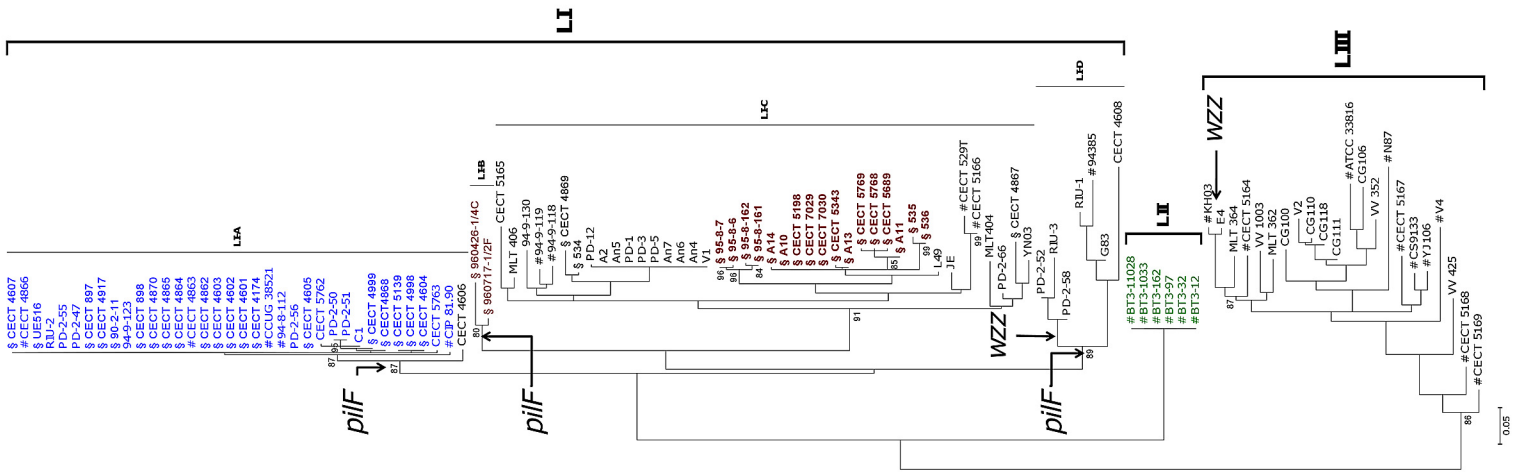

FIG. 1. Maximum likelihood phylogenetic tree of 115 *V. vulnificus* isolates obtained from the alignment of 7 concatenated loci. Black, biotype 1 isolates; blue, biotype 2 serovar E isolates; red, biotype 2 non-serovar E isolates; green, biotype 3 isolates; #, human isolates; §, diseased fish isolates. Branches where recombination events involving the indicated loci might have occurred are indicated by arrows. The numbers at the nodes represent the percentage values given by bootstrap analysis of 1,000 replicates.

TABLE 2. Genetic diversity parameters for the 4 housekeeping and 3 virulence-associated loci studied in 115 *V. vulnificus* isolates

Locus	Chromosome	Sequence length	No. of haplotypes	Haplotype diversity	Nucleotide diversity (π)	Polymorphic sites (S)	θ (from S)	Pairwise nucleotide differences (k)
mdh	I	489	32	0.82	$8.5 \cdot 10^{-3}$	23	0.01	4.2
pilF	I	480	27	0.87	$3.3 \cdot 10^{-2}$	57	0.025	15.72
wzz	I	460	33	0.84	$2.7 \cdot 10^{-2}$	61	0.027	12.65
glp	I	479	31	0.87	$1.8 \cdot 10^{-2}$	50	0.02	8.55
pyrC	II	423	31	0.79	$1.6 \cdot 10^{-2}$	39	0.02	6.99
pntA	II	396	24	0.85	$1.5 \cdot 10^{-2}$	33	0.018	5.95
vvhA	II	432	22	0.85	$1.8 \cdot 10^{-2}$	32	0.015	7.921
Value for:								
All genes		3,159	73	0.95	$2 \cdot 10^{-2}$	295	0.017	20.87
Chromosome I		1,908	66	0.94	$2.1 \cdot 10^{-2}$	191	0.02	41.11
Chromosome II		1,251	53	0.91	$1.6 \cdot 10^{-2}$	104	0.017	20.88
Housekeeping genes		1,787	68	0.95	$1.4 \cdot 10^{-2}$	145	0.016	25.69
Virulence genes		1,372	49	0.89	$2.6 \cdot 10^{-2}$	150	0.023	36.3

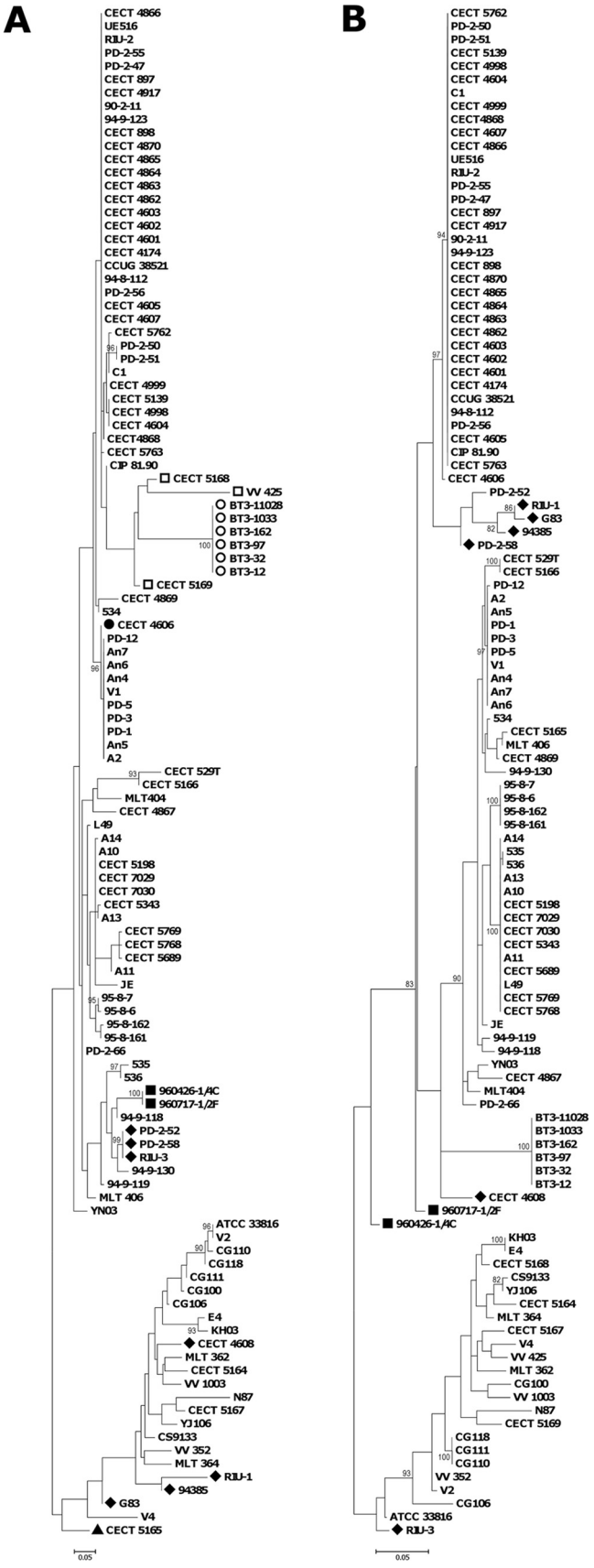

FIG. 2. Maximum likelihood phylogenetic tree of 115 *V. vulnificus* isolates obtained from the alignment of 4 housekeeping (A) and 3 virulence-associated (B) concatenated loci. Symbols indicate isolates that cluster in LI-A (●), LI-B (■), LI-C (▲), LI-D (◆), LII (○), or LIII (□) in the concatenated tree. The numbers at the nodes represent the percentage values given by bootstrap analysis of 1,000 replicates.

TABLE 3. Likelihood scores for the loci identified as involved in recombination events[a]

Alignment	−lnL for ML tree of the:	
	Locus	Concatenated loci
pilF	−3,136.12	−4,148.14*/*
Concatenated loci without *pilF*	−11,095.79*/*	−9,753.16
wzz	−3,125.20	−4,001.95*/*
Concatenated loci without *wzz*	−11,302.35*/*	−9,503.07

[a] For each recombinant locus, we considered two multiple alignments, the one from that locus and that obtained from the concatenated alignment of the other 6 loci. These two alignments were used to evaluate the likelihood (−lnL, negative natural log of the likelihood) of the ML trees obtained with each of them, and the tree obtained with each alignment is compared with that derived from the other by the Shimodaira-Hasegawa (SH) and expected likelihood weight (ELW) tests. Levels of significance for the SH/ELW tests: *, $P < 0.001$.

amplification after epizootics in fish farms. The exception would be the clone formed by biotype 3 isolates, the only ones capable of causing outbreaks of human vibriosis.

Furthermore, LI can be subdivided into four main groups, as follows: LI-A, grouping all biotype 2 serovar E isolates plus a Spanish biotype 1 isolated from a fish tank (CECT 4606); LI-B, formed by two atypical isolates of biotype 2 from Denmark; LI-C, clustering all non-serovar E biotype 2 isolates together with biotype 1 isolates from fish farms and humans; and lastly, LI-D, grouping biotype 1 isolates from the environment and humans (mainly from blood). Thus, biotype 2 is polyphyletic and appears to be divided into serovar-related subgroups, the isolates in each subgroup being more related to biotype 1 isolates from fish farms than to each other. This result is compatible with the hypothesis that biotype 2 emerged by acquisition of the virulence plasmid by *V. vulnificus* strains from fish farms.

The groupings of strains changed when only virulence or housekeeping genes were considered (Fig. 2). In both cases, the position of biotype 3 changed, and this also occurred in the individual gene trees (Fig. S1 to S5 in the supplemental material). Previous studies of the phylogeny of *V. vulnificus* (based on different techniques, multilocus enzyme electrophoresis, MLST, or 16S rRNA or gene sequencing) divided the species into two main lineages, the clinical and the environmental lineages (5, 6, 10, 15, 19, 23, 26). In our study, the division in the two lineages was observed in the phylogenetic trees from virulence-related or from housekeeping genes but not in the concatenated phylogenetic tree. Thus, it can be concluded that combining four housekeeping and three virulence gene sequences in the analysis gives enough resolution to show biotype 3 as an independent lineage. Bisharat et al. (3) proposed that biotype 3 contains a mosaic genome that would have evolved by hybridization of genomes of representative strains of the other two lineages. Our results sup

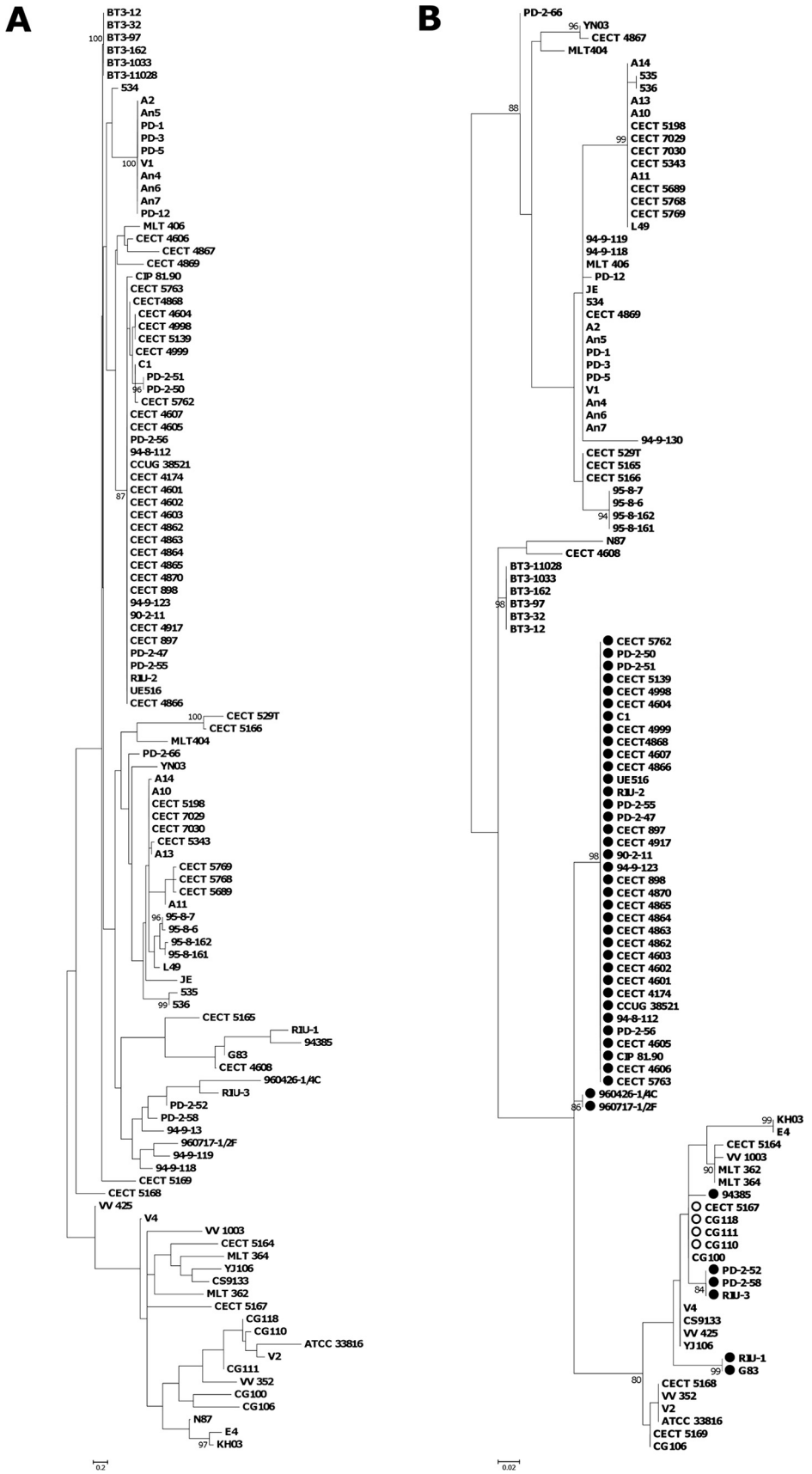

FIG. 3. Maximum likelihood phylogenetic trees from 6 of the 7 concatenated loci, excluding region *pilF* (A) and the *pilF* locus (B). Isolates identified as recombinants are marked with a filled circle, while the putative parental isolates are marked with an open circle. The numbers at the nodes represent the percentage values given by bootstrap analysis of 1,000 replicates.

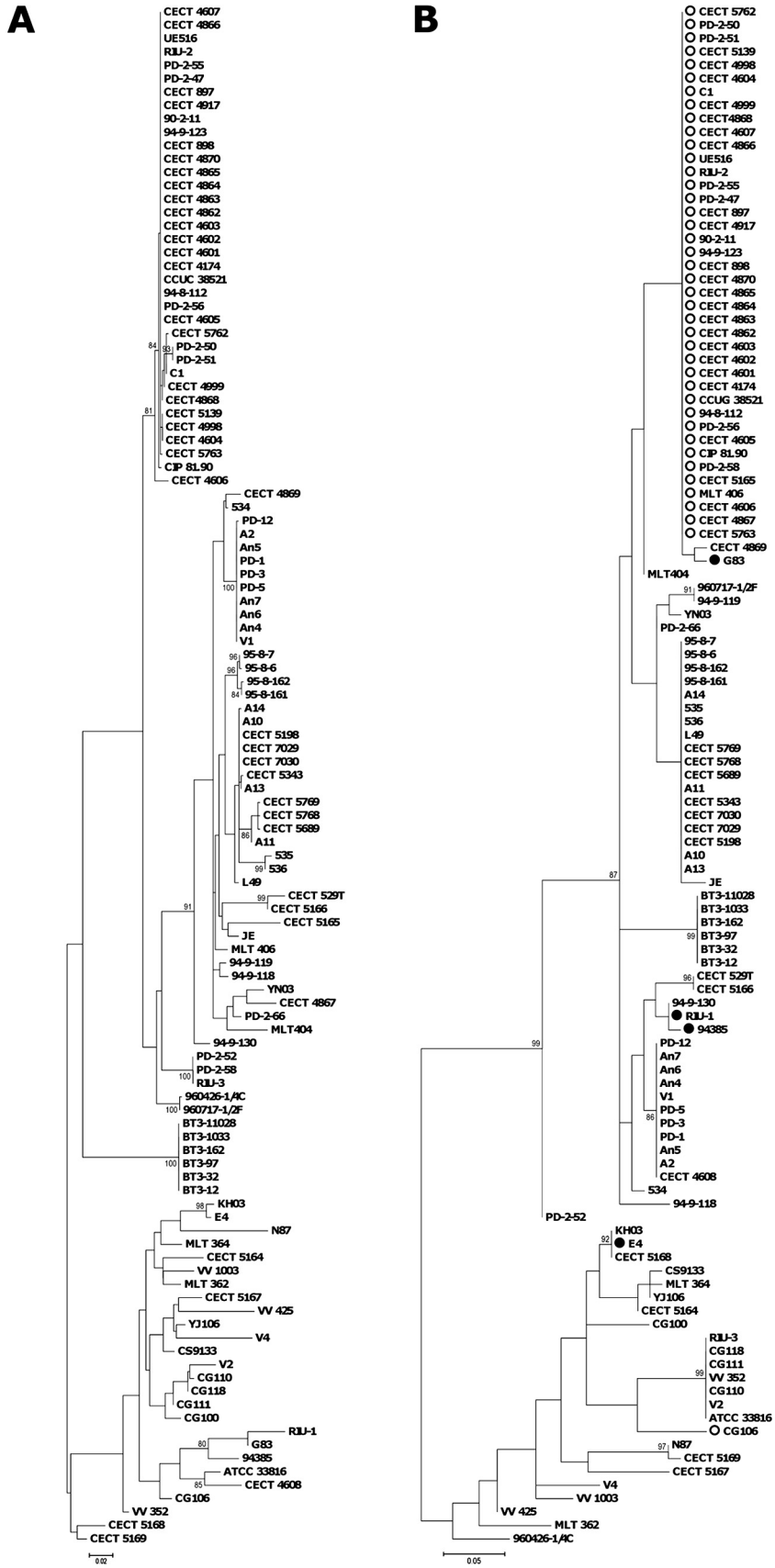

FIG. 4. Maximum likelihood (ML) phylogenetic trees from 6 of the 7 concatenated loci, excluding region *wzz* (A) and the *wzz* locus (B). Isolates identified as recombinants are marked with a filled circle, while the putative parental isolates are marked with an open circle. The numbers at the nodes represent the percentage values given by bootstrap analysis of 1,000 replicates.

port this hypothesis, although the change of position in the trees affected not only biotype 3 isolates but also biotype 2 non-serovar E isolates and biotype 1 strains, mostly from fish-farming environments (Fig. 2; also see Fig. S1 to S5 in the supplemental material).

The Recombination Detection Program, version 3 (RDP3) (14) detected recombination in chromosome I by at least four of the implemented methods, involving two of the four loci (*pilF* and *wzz*). The putative recombinant events were mapped onto the phylogenetic tree of the seven concatenated loci (Fig. 1). To further corroborate the RDP3 results, two different ML trees were constructed for each recombinant locus. The tree obtained from the multiple sequence alignment of each locus was compared with the one obtained from the concatenated sequences of all the other loci by using TreePuzzle version 5.2 (22). In both cases, Shimodaira-Hasegawa (SH) and expected likelihood weight (ELW) tests revealed significant differences between the two topologies (Table 3). Figures 3 and 4 each show the phylogenetic tree of one of the loci involved in recombination (*pilF* and *wzz*) and the tree derived from the remaining aligned loci. In both cases, biotype 2 serovar E strains are involved in the recombination events, either as predicted parental strains, in *wzz*, or as daughter strains, in *pilF*. The other strains involved in recombination events are biotype 2 nontypeable strains and clinical and environmental biotype 1 isolates. This result suggests that the conditions of aquaculture settings (e.g., high nutrient loads and high host density) might favor the exchange of genetic material among strains of *V. vulnificus*, originating new variants in the *V. vulnificus* species.

Interestingly, the *pilF* ML tree splits the strains into two groups, one that clusters most clinical biotype 1 isolates from humans together with biotype 3 and biotype 2 serovar E isolates from human origins and another that groups serovar A and I isolates together with environmental biotype 1 isolates (Fig. 3B), suggesting that *pilF* could be used as a genetic marker to distinguish isolates potentially dangerous to humans. A PCR-based protocol to distinguish *V. vulnificus* isolates with pathogenic potential against humans based on the polymorphism in *pilF* has been designed and validated in our laboratory (18).

Conclusion. The *V. vulnificus* species is subdivided into three different phylogenetic lineages which do not correspond to the current intraspecific biotype classification. LI and LII seem to have evolved in fish-farming-related environments where recombination or/and horizontal transfer phenomena would have favored the emergence of pathogenic clones for fish or humans, which would have been amplified after outbreaks of fish (biotype 2) or human (biotype 3) vibriosis. The polyphyletic origin of the so-called biotype 2 supports its reclassification within the species as a pathovar that would group the strains with pathogenic potential to infect and develop vibriosis in fish.

This work was financed by grants AGL2008-03977/ACU, BFU2008-03000, and Programa Consolider-Ingenio 2010 CSD2009-00006 from MICINN and by ACOMP/2009/240 from Conselleria d'Educació (Generalitat Valenciana).

We thank the SCSIE of the University of Valencia for technical support in determining the sequences.

REFERENCES

1. **Amaro, C., and E. G. Biosca.** 1996. *Vibrio vulnificus* biotype 2, pathogenic for eels, is also an opportunistic pathogen for humans. Appl. Environ. Microbiol. **62:**1454–1457.
2. **Bisharat, N., et al.** 2007. The evolution of genetic structure in the marine pathogen, *Vibrio vulnificus*. Infect. Genet. Evol. **7:**685–693.
3. **Bisharat, N., et al.** 2005. Hybrid *Vibrio vulnificus*. Emerg. Infect. Dis. **11:**30–35.
4. **Bisharat, N., 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. **Chatzidaki-Livanis, M., M. A. Hubbard, K. Gordon, V. J. Harwood, and A. C. Wright.** 2006. Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. Appl. Environ. Microbiol. **72:**6136–6141.
6. **Cohen, A. L., J. D. Oliver, A. DePaola, E. J. Feil, and E. F. Boyd.** 2007. Emergence of a virulent clade of *Vibrio vulnificus* and correlation with the presence of a 33-kilobase genomic island. Appl. Environ. Microbiol. **73:** 5553–5565.
7. **Fouz, B., and C. Amaro.** 2003. Isolation of a new serovar of *Vibrio vulnificus* pathogenic for eels cultured in freshwater farms. Aquaculture **217:**677–682.
8. **Guindon, S., and O. Gascuel.** 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52:**696–704.
9. **Gulig, P. A., K. L. Bourdage, and A. M. Starks.** 2005. Molecular pathogenesis of *Vibrio vulnificus.* J. Microbiol. **43**(Spec. No.)**:**118–131.
10. **Gutacker, M., et al.** 2003. Population genetics of *Vibrio vulnificus*: identification of two divisions and a distinct eel-pathogenic clone. Appl. Environ. Microbiol. **69:**3203–3212.
11. **Jones, M. K., and J. D. Oliver.** 2009. *Vibrio vulnificus*: disease and pathogenesis. Infect. Immun. **77:**1723–1733.
12. **Lee, C. T., et al.** 2008. A common virulence plasmid in biotype 2 *Vibrio vulnificus* and its dissemination aided by a conjugal plasmid. J. Bacteriol. **190:**1638–1648.
13. **Marco-Noales, E., M. Milan, B. Fouz, E. Sanjuan, and C. Amaro.** 2001. Transmission to eels, portals of entry, and putative reservoirs of *Vibrio vulnificus* serovar E (biotype 2). Appl. Environ. Microbiol. **67:**4717–4725.
14. **Martin, D. P., C. Williamson, and D. Posada.** 2005. RDP2: recombination detection and analysis from sequence alignments. Bioinformatics **21:**260–262.
15. **Nilsson, W. B., R. N. Paranjype, A. DePaola, and M. S. Strom.** 2003. Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. J. Clin. Microbiol. **41:**442–446.
16. **Posada, D., and K. A. Crandall.** 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14:**817–818.
17. **Roig, F. J., and C. Amaro.** 2009. Plasmid diversity in *Vibrio vulnificus* biotypes. Microbiology **155:**489–497.
18. **Roig, F. J., E. Sanjuan, A. Llorens, and C. Amaro.** 2010. *pilF* polymorphism-based PCR to distinguish *Vibrio vulnificus* strains potentially dangerous to public health. Appl. Environ. Microbiol. **76:**1328–1333.
19. **Rosche, T. M., Y. Yano, and J. D. Oliver.** 2005. A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. Microbiol. Immunol. **49:**381–389.
20. **Rozas, J., J. C. Sanchez-DelBarrio, X. Messeguer, and R. Rozas.** 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics **19:**2496–2497.
21. **Sanjuan, E., B. Fouz, J. D. Oliver, and C. Amaro.** 2009. Evaluation of genotypic and phenotypic methods to distinguish clinical from environmental *Vibrio vulnificus* strains. Appl. Environ. Microbiol. **75:**1604–1613.
22. **Schmidt, H. A., K. Strimmer, M. Vingron, and A. von Haeseler.** 2002. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics **18:**502–504.
23. **Senoh, M., et al.** 2005. The cytotoxin-hemolysin genes of human and eel pathogenic *Vibrio vulnificus* strains: comparison of nucleotide sequences and application to the genetic grouping. Microbiol. Immunol. **49:**513–519.
24. **Strom, M. S., and R. N. Paranjpye.** 2000. Epidemiology and pathogenesis of *Vibrio vulnificus*. Microb. Infect. **2:**177–188.
25. **Tison, D. L., M. Nishibuchi, J. D. Greenwood, and R. J. Seidler.** 1982. *Vibrio vulnificus* biogroup 2: new biogroup pathogenic for eels. Appl. Environ. Microbiol. **44:**640–646.
26. **Warner, J. M., and J. D. Oliver.** 1999. Randomly amplified polymorphic DNA analysis of clinical and environmental isolates of *Vibrio vulnificus* and other vibrio species. Appl. Environ. Microbiol. **65:**1141–1144.
27. **World Health Organization and Food and Agriculture Organization of the United Nations.** 2005. Risk assessment of *Vibrio vulnificus* in raw oysters. WHO Press, Geneva, Switzerland.