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Phenotypic and Genotypic Characterization of *Vibrio vulnificus*: Proposal for the Substitution of the Subspecific Taxon Biotype for Serovar

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The classification of *Vibrio vulnificus* strains into two biotypes has been maintained on the basis of phenotypic properties and eel virulence. Biotype 2 is virulent for eels, negative for the indole reaction, and serologically homogeneous (serogroup E), whereas strains of biotype 1 are avirulent, indole positive, and serologically heterogeneous. In the present study, we phenotypically and genotypically characterized 21 *V. vulnificus* isolates, recovered mainly from northern Europe, by comparing them with reference strains of both biotypes to look for new isolates of biotype 2. The results of this work revealed that the majority of isolates virulent for eels presented phenotypic traits previously considered characteristics of biotype 2 and specific ribotypes with *Hin*dIII. However, among the new isolates we found (i) a serogroup E strain virulent for eels but indole positive and (ii) one isolate not belonging to serogroup E but pathogenic for eels. Since no biochemical test or specific serogroup can with certainty be associated with eel virulence, we propose to classify *V. vulnificus* strains into serovars instead of biotypes. Thus, we suggest serovar E as the denomination of those strains previously classified as biotype 2. Finally, the occurrence of serogroup E in eels cultured in Norway and Sweden, as well as from human infections and shrimp, has been demonstrated.

Vibrio vulnificus is a pathogenic bacterial species which comprises phenotypically different strains that have been classified into two biological groups defined mainly by the indole test and host range (42). Biotype 1 is ubiquitous in the marine environment, where it can be spread and cause fatal human infections, but is avirulent for eels. In contrast, biotype 2 has been considered for a long time to be restricted to diseased eels. This biotype was first isolated in Japan, where it produced several epizootics in cultured eels between 1975 and 1977 (32–37). No reports were published in other countries until 1989, when we reported its isolation from diseased eels in Spain (10). Since then, biotype 2 was recovered during different outbreaks that occurred in the same farm (2, 16). Thus, we focused on the characterization of the Spanish biotype 2 isolates, using reference strains of both biotypes for comparison. Our investigations have revealed that biotypes 1 and 2 of *V. vulnificus* exhibit a high level of phenotypic homology, sharing many biochemical traits, immunologically related outer membrane proteins (OMPs), the expression of several virulence factors, and virulence for mice (2, 5, 9, 13–16). Further, we have demonstrated the survival of the eel pathogen in seawater as a free-living microorganism, as well as its water-borne transmissibility (3, 11). However, the two biotypes still can be differentiated based on biochemical and serological properties, as well as by eel pathogenicity (16). In this sense, we have recently proposed that (i) the indole reaction seems to be the only biochemical test distinguishing between biotypes 1 and 2, with the negative response for this test being characteristic of all biotype 2 isolates tested by us; (ii) biotype 2 appears to constitute a homogeneous lipopolysaccharide (LPS)-based O serogroup (serogroup E, from eels), while different O serovars have been observed among biotype 1 strains; and (iii) serogroup E comprises the strains of the species which are pathogenic for eels and harbor high-M_r plasmids (16). We have discovered that an indole-negative strain implicated in a clinical case actually belongs to biotype 2 (1). This clinical strain belongs to serogroup E, is virulent for eels, and harbors high-M_r plasmids (1). The occurrence of *V. vulnificus* in Europe has been described since 1979 (6, 17, 19, 22, 23, 25, 30, 31, 43–46), but isolation of biotype 2 has been reported only for eels cultured in Spain (2, 10, 16). However, eels have been implicated as vectors for some clinical *V. vulnificus* infections caused by indole-negative variants in northern Europe (31, 44–46). The recent report of indole-negative strains from water or sediment samples indicates that this biotype may also be found in other natural environments (19). The former could explain, at least in part, the lack of reports of biotype 2 from sources other than diseased eels.

There is a considerable number of reports about the isolation of *V. vulnificus* from both clinical and environmental sources worldwide, but usually no information about the biotype is provided. This fact impedes epidemiological studies of the eel pathogen. The aim of the present study was to determine the biotype of some unreported *V. vulnificus* isolates mainly from northern Europe to find new isolates of biotype 2. To this end, we serologically, biochemically, and molecularly characterized these isolates by using strains of both biotypes for comparison. Selected strains were further assayed for eel virulence, while all isolates were genotypically analyzed by ribotyping. In the course of the present investigation, we found exceptions to the traits that support the subdivision of the species in biotypes. As we detail in this article, we propose the classification of *V. vulnificus* in serovars.

MATERIALS AND METHODS

Bacterial strains. We employed 49 strains from different sources and origins, including 26 *V. vulnificus* strains of both biotypes, as well as 23 strains provisionally identified as *V. vulnificus* but without information about their biotype (Table 1). Bacterial strains were routinely grown in Luria broth (Difco) or Trypticase

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TABLE 1. Sources and origins of the *V. vulnificus* strains used in this study[a]

Strain[b]	Source	Country, yr	Colony hybridization	Serovar E	API 20 E profile	Indole test	ODC	Mannitol	Growth at 42°C	Plasmid size(s) (kb)	Virulence for eels	Biotype	Ribotype by *Hin*dIII
ATCC 33187	Leg wound	United States	+	+	5306005	−	+	−	−	76, 54	+	2	1
ATCC 33149	Diseased eel	Japan	+	+	5006005	−	−	−	−	110, 72, 54	+	2	1
NCIMB 2138	Diseased eel	Japan	+	+	5206005	−	−	−	−	70, 56	+	2	1
NCIMB 2137	Diseased eel	Japan	+	+	5006004	−	−	−	−	70, 54	+	2	1
NCIMB 2136	Diseased eel	Japan	+	+	4006005	−	−	−	−	76, 54	+	2	1
UE 516[c]	Diseased eel	Taiwan	+	+	5006005	−	−	−	−	72, 54	+	2	1
E22[d]	Diseased eel	Spain, 1989	+	+	5206005	−	−	−	−	72, 56	+	2	1
E39[d]	Diseased eel	Spain, 1990	+	+	5206005	−	−	−	−	72, 56	+	2	1
E58[d]	Diseased eel	Spain, 1990	+	+	5006005	−	−	−	−	72, 56	+	2	1
E86[d]	Diseased eel	Spain, 1990	+	+	5206005	−	−	−	−	72, 56	+	2	1
E105[d]	Diseased eel	Spain, 1990	+	+	5306005	−	+	−	−	72, 28	+	2	1
E116[d]	Diseased eel	Spain, 1992	+	+	5306005	−	+	−	−	72, 28	+	2	1
H2[e]	Diseased eel	Spain, 1994	+	+	5306005	−	+	−	−	72	+	2	2 ≈ 1
524[f]	Diseased eel	Norway, 1990	+	+	5106005	−	+	−	−	84, 54, 28	+	2	1
121[f]	Diseased eel	Sweden, 1991	+	+	5102004	−	+	−	−	74, 52	+	2	1
525[f]	Diseased eel	Sweden, 1991	+	+	5306004	−	+	−	−	74, 48	+	2	1
526[f]	Diseased eel	Sweden	+	+	5102005	−	+	−	−	74, 48	+	2	1
527[f]	Diseased eel	Sweden	+	+	5306005	−	+	−	−	80, 48	+	2	1
529[f]	Diseased eel	Sweden	+	+	5106005	−	+	−	−	74	+	2	1
171[f]	Diseased eel	Sweden, 1992	+	+	1006005	−	−	−	−	82, 54	+	2	1
522[f]	Human blood	Australia	+	+	5106005	−	+	−	+	74	+	2	1
520[f]	Shrimp	Taiwan	+	+	5004005	−	−	−	−	84, 62	+	2	1
523[f]	Diseased eel	Unknown	+	+	5146005	+	+	−	+		+	2	3 ≈ 1
ATCC 27562[T]	Human blood	United States	+	−	5146005	+	+	−	+		−	1	4
B9629[g]	Wound infection	United States	+	−	5146105	+	+	+	+		ND	1	4
C7184[g]	Human blood	United States	+	−	5346105	+	+	+	+		−	1	5
L-180[g]	Septicemia case	United States	+	−	5346105	+	+	+	+		ND	1	5
ATCC 33186	Human blood	United States	+	Atypical	5146105	+	+	+	−		−	1	6
ATCC 33184	Human blood	United States	+	−	5346105	+	+	+	+		ND	1	7
374[g]	Septicemia case	United States	+	−	5346105	+	+	+	+		ND	1	8
MO6-24[g]	Human blood	United States	+	−	4346105	+	+	+	+		ND	1	9
H3308[g]	Clinical sample	United States	+	−	5346105	+	+	+	+		ND	1	10
UMH1[g]	Wound infection	United States	+	−	5146105	+	+	+	−		ND	1	11
UNCC 890[g]	Environmental sample	United States	+	−	5146005	+	+	−	−		−	1	12
TW1[e]	Tank water	Spain, 1990	+	−	5146105	+	+	+	−	13, 7	−	1	13
821[f]	Brackish water	France, 1991	+	−	5146005	+	+	−	−		ND	1	14
528[f]	Diseased eel	Sweden	+	−	5146105	+	+	+	+	115, 72, 48	ND	1	15
534[f]	Diseased eel	Sweden	+	Atypical	5144105	+	+	+	−	115, 72, 49	−	1	16
535[f]	Diseased eel	Sweden	+	−	5146105	+	+	+	−	74	ND	1	17
536[f]	Diseased eel	Sweden	+	−	5146105	+	+	+	−	74	ND	1	17
530[f]	Diseased eel	Belgium, 1990	+	−	5146025	+	+	−	−	68, 50, 35, 10	+	1	18
532[f]	Diseased eel	Belgium, 1990	+	−	5046005	+	−	−	−		−	1	19
E109[d]	Healthy eel	Spain, 1990	+	−	5346005	+	+	−	−	67, 48	−	1	20
537[f]	Shrimp	Thailand, 1992	+	−	5146105	+	+	+	+		ND	1	21
519[f]	Shrimp	Taiwan	+	−	5146105	+	+	+	+		−	1	21
521[f]	Unknown	Australia	+	Atypical	5346005	+	+	−	+	35	−	1	22
628[f]	Paguara (fish)	Venezuela	−	−	5144127	+	+	+	−		ND	ND	A
818[f]	Unknown	France	−	−	4144125	+	+	+	−		ND	ND	B
822[f]	Shrimp	Senegal, 1980	−	−	5044105	+	−	+	−	33	ND	ND	C

[a] Serogroup E was determined by Western blotting with antisera against cells of *V. vulnificus* biotype 2 strain E22. Colony hybridization, API 20E profiles, plasmid contents, eel virulence, biotype, and ribotypes of the strains were studied. ND, not done.

[b] ATCC, American Type Culture Collection, Rockville, Md. CDC, Centers for Disease Control and Prevention, Atlanta, Ga. FDA, Food and Drug Administration, Cincinnati, Ohio. NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland. T, type strain.

[c] Supplied by Y. L. Song, National Taiwan University, Taipei, Taiwan.

[d] Biosca et al. (10–16).

[e] Supplied by E. Alcaide, Universidad de Valencia, Valencia, Spain.

[f] Presumptive *V. vulnificus* strain without reference to biotype. Supplied by Inger Kühn, Karolinska Institute, Stockholm, Sweden; Linda Verdonck, Rijksuniversiteit, Gent, Belgium; Steinar Høie, Veterinærinstituttet, Oslo, Norway; Brian and Dawn Austin, Heriot-Watt University, Edinburgh, Scotland; Inger Dalsgaard, Danish Institute of Fisheries Research, Copenhagen, Denmark; or Patrick Grimont, Institut Pasteur, Paris, France.

[g] Supplied by J. D. Oliver, University of North Carolina, Charlotte.

soy agar (Difco) supplemented with 0.5% (wt/vol) NaCl. Isolates were incubated at room temperature (around 25°C), unless otherwise indicated.

Colony hybridization. Isolates were assayed by colony hybridization experiments using a digoxigenin-labeled cDNA probe complementary to rRNA of *V. vulnificus* (19-base DNA oligonucleotide Vvu2) (7). The probe was labeled with digoxigenin (Boehringer GmbH, Mannheim, Germany) by the 3′-end-labeling method using a kit obtained from Promega Corporation (Madison, Wis.). Hybridizations were performed as previously described (28), except that they were done at 46°C. Development conditions were the same as those used before for ribotyping (38). Strains were assayed in duplicate on separate filters. The positive

and negative controls used were *V. vulnificus* biotype 1 strain ATCC 27562 and a *V. cholerae* non-O1 strain, respectively.

Serological characterization. Whole cells and thermostable O antigens from all isolates were tested by slide agglutination with rabbit antisera against whole cells of biotype 2 (serogroup E, from eels) strains NCIMB 2137 and E22 and crude LPS of strain E22 as previously described (2, 16). Further, LPS and OMPs were also characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis combined with immunoblotting. OMPs were obtained as described by Biosca et al. (14). LPS extracts were prepared from whole-cell lysates by a modification of the method of Hitchcock and Brown (24) as previously described (4). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of OMP and LPS samples was done by the Laemmli method (27) as described before (4, 14). OMP and LPS bands were visualized by immunoblotting with antisera raised against biotype 2 cells as previously described (4, 14). Protein bands were also stained with Coomassie brilliant blue R-250.

Biochemical characterization. The commercial miniaturized API 20E system (BioMérieux, Marcy-l'Etoile, France) was used for presumptive identification and biotyping of isolates as previously described (12). Incubations were done at 25°C, and readings were performed after 24 and 48 h. Profiles were checked against those in the API index. The oxidase reactions were performed separately from the API 20E gallery. Conventional biochemical tests were carried out in parallel by previously described procedures (10, 16). Additionally, the following biochemical tests were performed: oxidative/fermentative (O/F) metabolism, growth in 0 and 6% NaCl, growth at 42°C, and sensitivity to the vibriostatic agent O/129 (150 μg).

Plasmid profiling. Extraction of plasmid DNA was performed by the method of Kado and Liu (26). Samples were immediately electrophoresed as previously described (15, 16). Molecular sizes were estimated by using several reference plasmids from *Escherichia coli* V517 (ranging from 54 to 2 kb), *E. coli* 39R861 (ranging from 147 to 6.9 kb), and *V. anguillarum* 775 (67 kb). All isolates were screened several times.

Virulence assays. Assays for pathogenicity were done with juvenile European eels (7 to 15 g, average) by intraperitoneal inoculation as previously described (2, 15, 16).

Ribotyping. Chromosomal DNA used for ribotyping was extracted essentially by the method of Pedersen and Larsen (38). Preliminary DNA digestions were carried out with selected strains by using *Hin*dIII, *Bgl*I, *Kpn*I, and *Mlu*I purchased from Promega. Genomic DNA was digested as recommended by the manufacturer. The DNA restriction fragments were separated by electrophoresis in 0.8% (wt/vol) agarose gels with TAE electrophoresis buffer as described before (38). The blotting procedure, hybridization with a digoxigenin-labeled cDNA probe complementary to the 16S and 23S rRNAs of *E. coli*, and development were carried out as previously described (38). A 1-kb DNA ladder (Boehringer) was used as a molecular weight marker. Ribotype patterns were considered to be different when there was a difference of one band between isolates. Each ribopattern was assigned an arbitrary number. Each isolate was ribotyped a minimum of three times.

RESULTS AND DISCUSSION

Colony hybridization. Colony hybridization experiments using an oligonucleotide probe specific for *V. vulnificus* (7) correctly differentiated reference *V. vulnificus* strains from the *V. cholerae* strain used as a negative control. Hence, this oligonucleotide was used for definitive identification of the new isolates tentatively classified as *V. vulnificus*. Results revealed that all of the new strains hybridized with this rRNA probe, producing a strong signal (Table 1), and were therefore identified as *V. vulnificus*, except for isolates 818, 822, and 628. These three strains gave a reaction similar to that observed with the negative control and were considered non-*V. vulnificus* strains (Table 1). These results indicated that *V. vulnificus* is more prevalent in Europe than previously reported, including environmental isolates from France, Belgium, Norway, and Sweden.

Serological characterization. We have already proposed that *V. vulnificus* biotype 2 constitutes a homogeneous O serogroup (serogroup E) based on the serological specificity of its LPS (16). Serogrouping was carried out preliminarily by slide agglutination using rabbit antisera against *V. vulnificus* biotype 2 strains E22 and NCIMB 2137. Among all of the new isolates, only strains 523, 524, 525, 526, 527, 529, 121, and 171, recovered from diseased eels mainly from Norway and Sweden; Taiwanese strain 520 from shrimp; and Australian isolate 522 from human blood showed a positive reaction with these

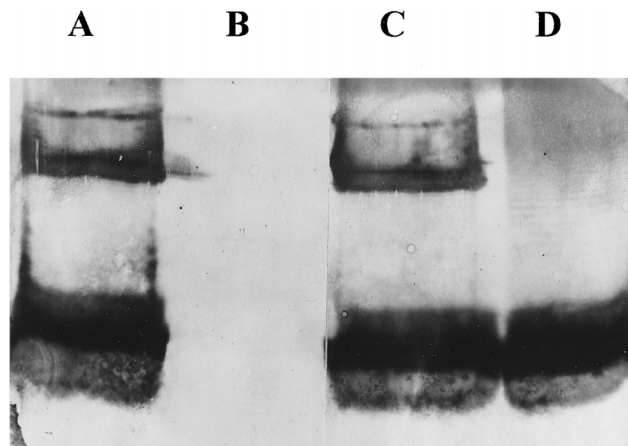

FIG. 1. LPS profiles of *V. vulnificus* isolates 534 (A), 530 (B), 521 (C), and 523 (D). LPS were extracted and immunostained with rabbit antiserum against serogroup E strain E22 as described by Amaro et al. (4).

antisera and were considered to belong to serogroup E (Table 1). The remaining isolates did not agglutinate with these antisera, except for clinical strain ATCC 33186 and environmental isolates 521 and 534, which exhibited cross-reactions, even with crude LPS antiserum. Consequently, all isolates were further analyzed by Western blotting with biotype 2 antisera. The immunological staining of LPS preparations from those isolates tentatively classified as serogroup E revealed a banding pattern characteristic of strains belonging to this serogroup (Table 1). In contrast, none of the LPS samples from the rest of the isolates reacted with these antisera, with the exception of those exhibiting cross-agglutinating properties (Table 1). In fact, the diffuse, fast-migrating band corresponding to the lipid A and core region of isolates ATCC 33186, 521, and 534 was stained with anti-serogroup E sera, with the last two strains also showing cross-reactivity in some portions of the O side chain of the LPS (Fig. 1). We have previously demonstrated that the two biotypes of *V. vulnificus* possessed serologically related OMPs and all of the biotype 2 strains tested by us showed similar protein profiles (14, 16). The OMPs of selected isolates, including serogroup E strains (E22, ATCC 33149, 520, 529, and 523), non-serogroup E strains (530), and those showing LPS portions antigenically related to this serogroup (521, 534, and ATCC 33186), were also analyzed by immunostaining with polyclonal antiserum against biotype 2 and stained with Coomassie brilliant blue. The OMP profiles of serogroup E isolates after Coomassie staining were very similar to those of the reference biotype 2 strains, while the rest of the isolates showed some differences, depending on the strain analyzed (data not shown), which is in agreement with our previous reports (14). The immunoblots stained with biotype 2 antisera revealed similar immunoreactive OMPs for all strains, with isolates 521 and 534 additionally presenting distinctive major proteins of around 58 and 64 kDa, respectively (Fig. 1 and 2). Interestingly, these two OMPs were resistant to the proteinase K treatment used for LPS preparation.

According to our previous reports, isolates belonging to serogroup E should be considered biotype 2 strains while non-serogroup E strains should be classified as biotype 1 strains (Table 1). Special attention should be paid to strains showing LPS portions antigenically related to serogroup E but harboring different thermolabile antigens. Further serological characterization is necessary to clarify the relationship of these strains with serogroup E. We consider these isolates to be

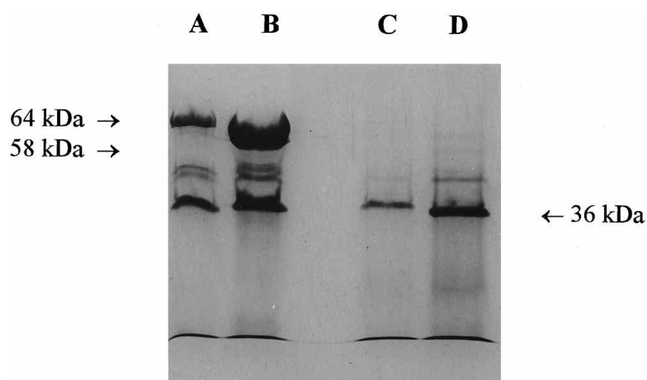

FIG. 2. OMP profiles of *V. vulnificus* isolates 534 (A), 521 (B), 530 (C), and 523 (D). OMPs were extracted and stained as described by Biosca et al. (14).

atypical serogroup E isolates (Table 1) whose biotype needs to be determined. Finally, serogrouping has confirmed the high prevalence of *V. vulnificus* isolates belonging to serogroup E in diseased eels.

Biochemical characterization. According to previous studies, the O/F test and growth in 0% NaCl were sufficient to confirm the identification of most *V. vulnificus* isolates when the API 20E system was used (12). However, in some cases, colony hybridization was required for definitive classification of both *V. vulnificus* and non-*V. vulnificus* strains. We have recently proposed that the indole reaction seems to be the only biochemical trait distinguishing *V. vulnificus* biotypes (16). This test is responsible for the typical profiles of biotype 2 in the API 20E system (12). After API 20E characterization, most of the serogroup E strains showed profiles characteristic of biotype 2 (Table 1). Some exceptions were observed: strain 523, which was positive for the indole reaction (Table 1), and strain 171, which lacked lysine decarboxylase activity (Table 1). All of these biotype 2 isolates were negative for mannitol and sorbitol fermentation, growth in 0% NaCl, and resistance to O/129 and were variable for ornithine decarboxylase (ODC) activity and growth in 6% NaCl (Table 1). These observations are in accordance with previous studies (10, 16). Interestingly, some biotype 2 isolates grew at 42°C, irrespective of their clinical or environmental origin (Table 1). The isolation of indole-negative strains, from both diseased eels and a case of human infection, able to grow at 42°C has previously been reported, but no reference to their biotype was provided (44). The rest of the strains, tentatively considered to belong to biotype 1, exhibited the API 20E profiles usually associated with this biotype (12). Interestingly, in some cases, indole detection was dependent on the medium employed since some strains that were indole negative in SIM agar (Difco) were positive in the API 20E system and in Luria broth, which is in agreement with previous reports (21, 40). Atypical serogroup E isolates 521 and 534 and strain ATCC 33186 were all indole positive, and the last two were also positive for mannitol, a biochemical trait so far associated only with biotype 1 (Table 1). Thus, they were preliminarily considered to belong to biotype 1. Biotype 1 isolates were negative for acid production from sorbitol, growth in 0% NaCl, and resistance to O/129, showing a variable response for ODC activity, acid production from mannitol, and growth in 6% NaCl and at 42°C, regardless of their origin. It is noteworthy that one isolate, strain 530, was positive for sucrose fermentation. The ability of *V. vulnificus* to ferment this sugar has been reported before (19, 41, 48).

All of these results indicate the limited value of biochemical tests for the identification of *V. vulnificus* and the diagnosis of vibriosis caused by biotype 2. In fact, the present work has demonstrated that biotype 2 may have the same API 20E profile as some biotype 1 isolates (Table 1). However, biochemical tests may still be valuable for further typing of isolates.

Plasmid profiling. In previous reports, we have demonstrated that biotype 2 strains contain high-M_r plasmids (15, 16). In the present study, the majority of biotype 2 strains harbored extrachromosomal DNA, with the plasmid profiles for most of the strains being similar to those previously reported (15, 16). All strains carried a common band of approximately 72 to 74 kb, which showed slight variation in size among some isolates (Table 1 and Fig. 3). Most of these isolates also harbored other plasmids whose masses ranged from 28 to 62 kb (Table 1 and Fig. 3). Some strains harbored more plasmids (Table 1). In contrast to previous reports (16), plasmid profiles did not allow us to distinguish ornithine-positive strains from ornithin-negative strains or to group isolates on the basis of geographical origin or source (Table 1 and Fig. 3). However, further studies including restriction analysis of extrachromosomal DNA are in progress to assess the value of plasmids for epidemiological studies of biotype 2. Surprisingly, no plasmid was detected after several extractions of biotype 2 strain 523 (Table 1). It is possible that this isolate lost plasmids after subculturing or

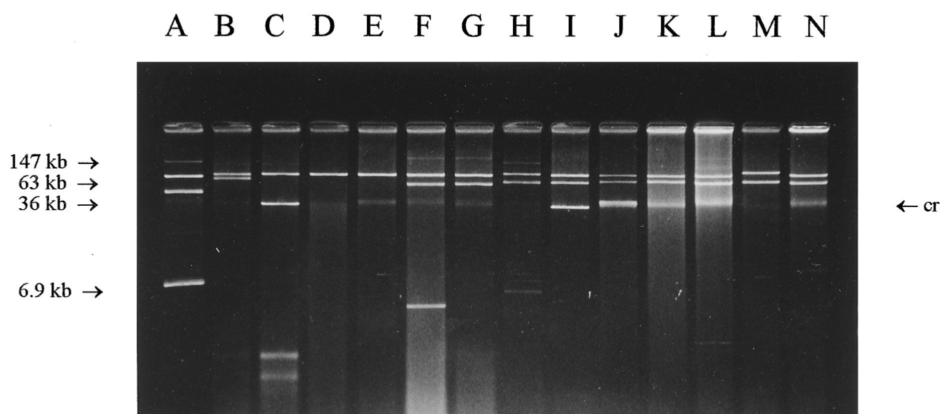

FIG. 3. Plasmid profiles of *E. coli* 39R 861 (A), and *V. vulnificus* E86 (B), E105 (C), H2 (D), 529 (E), 527 (F), 525 (G), 171 (H), 524 (I), ATCC 33149 (J), NCIMB 2138 (K), NCIMB 2137 (L), NCIMB 2136 (M), and UE516 (N). Plasmids were extracted by the method of Kado and Liu (26). cr, chromosome.

TABLE 2. Virulence of selected *V. vulnificus* strains for elvers

Strain	Challenge dose (CFU)	% Mortality	Red swelling at injection site
Biotype 2			
NCIMB 2136	4.8×10^4	66.6	+
E86	5.5×10^3	100	+
H2	6.0×10^4	83.3	+
UE516	5.4×10^4	83.3	+
520	8.9×10^4	100	+
522	8.0×10^4	100	+
524	1.7×10^5	100	+
525	1.0×10^5	83.3	+
526	5.5×10^4	83.3	+
527	3.4×10^5	100	+
529	4.6×10^4	66.6	+
121	4.1×10^4	83.3	+
171	1.3×10^5	100	+
523	5.0×10^7	83.3	+
523	4.6×10^6	0	+
Biotype 1			
ATCC 27562	1.4×10^8	0	−
ATCC 33186	8.7×10^7	0	−
E109	7.0×10^7	0	−
TW1	6.0×10^7	0	−
530	7.7×10^6	100	+
530	8.7×10^4	0	−
532	1.65×10^8	0	−
519	8.1×10^7	0	−
521	1.25×10^8	16.6	−
521	1.25×10^7	0	−
534	1.4×10^8	16.6	−
534	1.4×10^7	0	−

during storage. No reports on the stability of plasmids in *V. vulnificus* exist.

Previous studies have shown that most of the biotype 1 strains had no plasmids (16, 20). All of the North American strains were also plasmid free, including atypical serogroup E strain ATCC 33186. By contrast, the majority of European isolates, as well as one from Australia, presented extrachromosomal DNA. With the exception of strain 532, only those recovered from eels contained a high-molecular-weight plasmid band (Table 1). Whether this high-M_r plasmid is similar to or different from the one observed in biotype 2 isolates or if any relationship exists with eel pathogenicity is under investigation. It is also interesting that isolates 521 and 534, which expressed O-polysaccharide chains serologically related to serogroup E, showed extrachromosomal DNA (Table 1). The basic information for the LPS structure of gram-negative bacteria is usually chromosomal, but the existence of plasmids able to change the O side-chain structure is also well documented (39, 47). This could explain why some biotype 1 strains harboring plasmids shared some LPS compounds with serogroup E strains.

Eel pathogenicity. We have previously reported that the virulence mechanisms of the two biotypes of *V. vulnificus* exhibit a high degree of similarity and that both are virulent for mice and humans (1, 5, 9, 13, 15). However, only strains of biotype 2 are pathogenic for eels (2, 9, 13, 15, 16). The present virulence study has confirmed that all serogroup E strains were pathogenic for eels (Tables 1 and 2), with only those isolates carrying the large plasmid being highly virulent (Table 2). Bacteria were recovered in pure culture from internal organs of dead or moribund fish and subsequently identified by plasmid profiling. As expected, plasmid-free serogroup E strain 523 was pathogenic for eels, but doses as high as 10^7 cells/fish were necessary to produce infection in more than 50% of the fish tested, with deaths beginning after 48 to 72 h. Thus, we considered this strain to have a low level of virulence. Interestingly, this strain lacked the high-M_r plasmid typical of the rest of the biotype 2 strains, which suggests a relationship between this plasmid and eel virulence. Similar results have been described for *V. anguillarum* serogroup O1 strains cured from virulence plasmid pJM1 (18). Further studies including characterization of virulence properties of this strain, as well as pathogenicity assays using cured derivative strains, are necessary to verify this hypothesis.

Atypical serogroup E isolates (ATCC 33186, 521, and 534) and non-serogroup E strains 530, 532, and 519, all classified by us as biotype 1, were assayed for eel pathogenicity. Among all of these isolates, only non-serogroup E strain 530 was pathogenic for eels, with a lethal dose (10^6 cells/fish) (Table 2) higher than those displayed by most biotype 2 strains, so it was considered weakly virulent. This result, together with the fact that this isolate was recovered from the kidney of a diseased eel, indicates the existence of other serogroups pathogenic for eels within *V. vulnificus*. Interestingly, atypical serogroup E strains were avirulent for eels (Table 2). Because these strains also presented some OMPs different from those expressed by serogroup E isolates and since no studies of their virulence properties have been done, further investigations are necessary to explain their lack of virulence for eels. We believe that the lack of pathogenicity of atypical serogroup E isolates for eels supports their classification as biotype 1.

After the phenotypic characterization of the new isolates, it was evident that no biochemical test or specific serogroup can with certainty be associated with eel pathogenicity, which suggests that the classical subdivision of the species into biotypes cannot be maintained. However, before any conclusion could be reached, we considered it necessary to investigate the genetic homology of isolates virulent for eels.

Ribotyping. It has been proposed that *V. vulnificus* biotypes are genotypically distinguishable by ribotyping (8). Therefore, this technique was applied to all isolates to investigate the genetic homology of isolates, especially those virulent for eels. Ribotyping with *Hin*dIII was selected, since the ribopatterns obtained showed good separation of the resulting fragments. Among 46 *V. vulnificus* isolates, including reference strains, 22 ribotypes were observed (Table 1), with a group of four bands (ranging from 2.3 to <3 kb) being present in all of the *V. vulnificus* strains assayed (Fig. 4 and 5). As expected, the ribotypes obtained with non-*V. vulnificus* strains 818, 822, and 628 were quite different (data not shown) from those obtained with isolates belonging to this species. Thus, these results were consistent with those of the colony hybridization experiments, which demonstrated that the ribopatterns obtained with *Hin*dIII were species specific. On the basis of the hybridization bands observed, all but one of the isolates pathogenic for eels were identical or nearly identical (Fig. 4); all of these belonged to serogroup E, whereas the rest of the strains, including the non-serogroup E isolate virulent for eels (strain 530), were clearly different (Fig. 5). This indicates that pathogenicity for eels is not associated with a specific ribotype. The most characteristic feature of serogroup E ribotypes was the presence of two groups of two bands each (ranging from more than 3 to 4 kb) (Fig. 4), irrespective of the source and geographical origin. These results indicate a low level of genetic variation among serovar E isolates over time.

The ribopatterns of the remaining strains were more heterogeneous, but eight isolates, in four pairs, shared the same ribotype (Fig. 5). These isolates were the following: ATCC

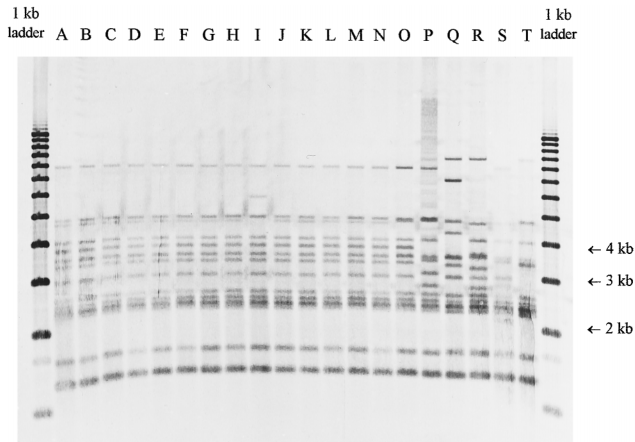

FIG. 4. Ribotypes of *V. vulnificus* strains. Lanes A to O, biotype 2 strains ATCC 33187 (A), 522 (B), ATCC 33149 (C), NCIMB 2138 (D), NCIMB 2137 (E), UE 516 (F), E22 (G), E105 (H), H2 (I), 524 (J), 121 (K), 171 (L), 529 (M), 520 (N), and 523 (O). Lanes P to T, biotype 1 strains 534 (P), 521 (Q), 530 (R), ATCC 27562 (S), and E109 (T). Ribotyping was performed as described by Pedersen and Larsen (38).

27562 and B9629, CDC 7184 and L-180, 535 and 536, and 519 and 537 (Table 1). The rest of the strains showed unique ribotypes. Despite the diversity observed in these ribotypes, the strains that shared the same ribotype were epidemiologically related, since the groups were formed by clinical or environmental strains, but none of the clinical isolates shared the ribotype with the environmental strains. Further studies including cluster analysis of the ribotypes observed are in progress to determine the value of ribotyping for epidemiological studies of this species. However, based on our present results, ribotyping with *Hin*dIII seems to be a powerful tool for both identification and typing of *V. vulnificus*.

The classification of *V. vulnificus* strains into two biotypes has been based on biochemical and serological differences that have been correlated with eel pathogenicity (1, 16, 42). Results from this work have revealed that most isolates virulent for eels presented phenotypic traits previously considered characteristics of biotype 2, which was correlated with the possession of specific ribotypes obtained with *Hin*dIII. However, among the new isolates, we found a serogroup E strain that was virulent for eels but indole positive and one isolate that did not belong to serogroup E but was pathogenic for eels. Since no biochemical test or specific serogroup can with certainty be associated with virulence for eels, the subdivision of this species into biotypes cannot be maintained. Based on the fact that all of the strains considered to belong to biotype 2 in this study belonged to the same LPS-based O serogroup (serogroup E), together with the previous detection of different *V. vulnificus* O serovars among biotype 1 strains (4, 16, 29), we propose to classify the strains of the species into serovars. Of these serovars, serovar E would contain the strains previously classified as biotype 2. Finally, we stress the high level of genetic homology observed among serovar E isolates, which can be isolated from a broader spectrum of samples and have a broader geographical distribution than previously reported. However, the high prevalence of strains belonging to serovar E in diseased eels suggests that this serogroup is specially adapted to eels.

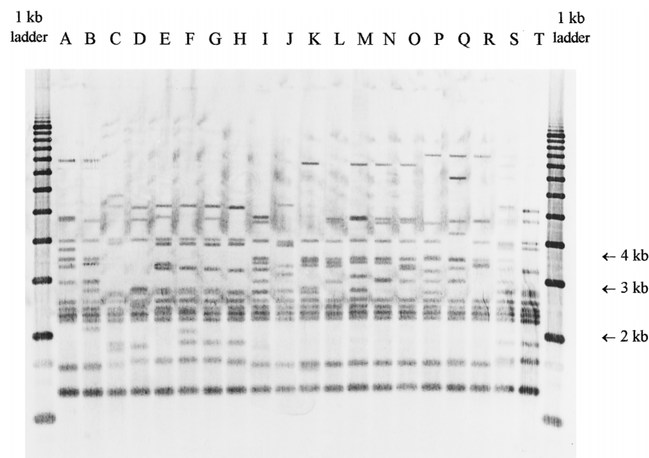

FIG. 5. Twenty different ribotypes of the 22 detected among 46 *V. vulnificus* strains. Lane A, biotype 2 strain 523. Lanes B to T, biotype 1 strains ATCC 27562 (B), CDC-7184 (C), ATCC 33186 (D), ATCC 33184 (E), 374 (F), MO6-24 (G), H3308 (H), UMH-1 (I), TW1 (J), UNCC 890 (K), 821 (L), 534 (M), 535 (N), 528 (O), 530 (P), 521 (Q), E109 (R), 532 (S), and 537 (T).

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