Plasmid diversity in *Vibrio vulnificus* biotypes

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Vibrio vulnificus is a heterogeneous bacterial species that can be virulent for humans and fish. Virulence in fish seems to rely on a recently described plasmid that can be transmitted between strains, aided by a conjugative plasmid. The main objective of this work was to analyse the plasmid content of a wide collection of strains from the three biotypes of the species, as well as to identify putative conjugative and virulence plasmids by means of Southern hybridization with specific probes and sequence analysis of selected gene markers. We found 28 different plasmid profiles in a total of 112 strains, which were relatively biotype- or serovar-specific. Biotype 1 lacked high-molecular-mass plasmids, with the exception of a putative conjugative plasmid of 48 kb that was present in 42.8 % of clinical and environmental strains isolated worldwide. All biotype 2 strains possessed the virulence plasmid, whose molecular mass ranged between 68 and 70 kb, and 89.65 % of these strains also had a putative conjugative plasmid with a molecular size of 52–56 kb. Finally, a 48 kb putative conjugative plasmid was present in all biotype 3 strains. Data from partial sequencing of *traD*, *tral* and the whole *vep07* (a recently described plasmid-borne virulence gene) from a selection of strains suggest that the plasmids of 48–56 kb probably belong to the same family of F-plasmids as pYJ016 and that the gene *vep07* is absolutely essential for fish virulence. Additional cryptic plasmids of low molecular mass were present in the three biotypes. In conclusion, plasmids are widespread among *V. vulnificus* species and could contribute substantially to genetic plasticity of the species.

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INTRODUCTION

Vibrio vulnificus is a heterogeneous aquatic species that has been subdivided into biotypes and serovars (Tison *et al.*, 1982; Bisharat *et al.*, 1999, 2007b). Biotypes 1 and 2 include environmental and clinical isolates recovered worldwide, while biotype 3 comprises solely clinical isolates from Israel (Oliver, 2006). Biotypes 1 and 2 are serologically diverse, whereas biotype 3 is serologically homogeneous (Amaro *et al.*, 1992; Bisharat *et al.*, 2007a, b; Fouz *et al.*, 2007). Three O-serovars have been described in biotype 2 (serovar A, serovar E, serovar I), one in biotype 3 (serovar O), and more than four in biotype 1, which has not been fully serotyped (Biosca *et al.*, 1996; Fouz *et al.*, 2007; Bisharat *et al.*, 2007a; Amaro *et al.*, 1992).

The three biotypes of *V. vulnificus* can cause disease in humans following ingestion of contaminated seafood or immersion exposure of an open wound to contaminated seawater (Oliver, 2005, 2006). In relatively healthy people, ingestion of or exposure to *V. vulnificus* leads to vomiting, diarrhoea and wound infection or severe ulceration, while in immunocompromised individuals, particularly those

with chronic liver disease, the pathogen can invade the bloodstream and cause septicaemia and death in almost 50 % of cases (Oliver, 2005, 2006). Biotype 2 also causes a disease called warm-water vibriosis in fish (mainly eels) and shrimp (Tison *et al.*, 1982). The strains of this biotype with pathogenic potential for humans belong to serovar E (Amaro & Biosca, 1996).

Recently, the genomes of two human biotype 1 isolates from Asia have been sequenced in Taiwan and South Korea (Chen *et al.*, 2003; Kim *et al.*, 2003). A comparison of these genomes revealed that the two strains share approximately 98 % of the chromosomal information, differing mainly in their plasmid content. For example, one of the strains (strain YJ016) possesses a conjugative plasmid of 48 kb, which is absent in the other isolate (Chen *et al.*, 2003; Kim *et al.*, 2003). Previous studies on plasmid carriage in *V. vulnificus* found these extrachromosomal elements to be rare within the species (Davidson & Oliver, 1986), with the exception of biotype 2 isolates, which appear to possess plasmids of high molecular mass (Biosca *et al.*, 1996; Lee *et al.*, 2008; Lewin *et al.*, 2000). One of these plasmids, 68 kb in size, seems to be a virulence plasmid essential for fish vibriosis since inactivation in one serovar E isolate resulted in a loss of resistance to eel serum, and thus was avirulent for eels (Lee *et al.*, 2008; Valiente *et al.*, 2008). This plasmid can be transmitted between strains, aided by a conjugative

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitro blue tetrazolium chloride; SSC, citrate buffer.

The GenBank/EMBL/DBJ accession numbers for the sequences resulting from the PCRs in this study are FJ000003–FJ000061.

plasmid of 56 kb, by cointegrate formation (Lee *et al.*, 2008). The virulence plasmids of two biotype 2 serovar E strains show 92 % homology (Lee *et al.*, 2008) and contain two sequences, *seq25* and *seq51*, that have previously been found, by subtractive hybridization, to be specific to eel-virulent strains and are associated with *vep07*, an ORF with an essential role in eel virulence (mutations in this gene produce the same effect as inactivation of the plasmid) (Lee *et al.*, 2008). This ORF codes for a novel protein with low similarity to a toxin of the family of RTX toxins (Lee *et al.*, 2008).

The aim of this work was to study the distribution of the virulence and conjugative plasmid, together with cryptic plasmids, among *V. vulnificus* biotypes and serovars, using probes derived from selected genetic markers for virulence and conjugative plasmids. The *vep07* ORF was sequenced in a selection of virulence plasmids and the sequences were compared in order to obtain information on the evolution of the virulence plasmid within this species.

METHODS

Bacterial strains and growth conditions. The *V. vulnificus* strains used in this study are listed in Table 1. *Escherichia coli* was routinely grown in Luria–Bertani broth (LB) or agar (LBA) (Pronadisa) and *V. vulnificus* strains were grown in Tryptone Soy Broth or agar plus 5 g NaCl l⁻¹ (TSB-1 or TSA-1, Pronadisa) at 28 °C for 24 h. For plasmid extraction, bacteria were grown in LB or TSB-1 for 4–6 h, and for genomic DNA extraction, they were grown overnight, in both cases with shaking at 28 °C. The strains were maintained both as lyophilized stocks and as frozen stocks at –80 °C in marine broth (Difco) plus 20 % (v/v) glycerol.

DNA extractions and electrophoretic analysis. Genomic DNA was extracted according to the mini-prep protocol for genomic DNA extraction (Ausubel *et al.*, 2007). Plasmid DNA was extracted by following the TENS protocol (Zhou *et al.*, 1990) with minor modifications, which included using a volume of 200 µl sodium acetate (3 M, pH 4.8) followed by a 10 min incubation on ice and reduction of the volume and number of phenol/chloroform/isoamyl alcohol (25:24:1) extractions. After dissolving DNA at 37 °C for 2 h, samples were subjected to electrophoresis on 0.7 % (w/v) agarose gels (Roche, molecular grade) for 3 h at 50 V. Plasmid molecular sizes were estimated using several reference plasmids: *E. coli* V517 (plasmids of 54.38, 7.30, 5.56, 5.14, 3.98, 3.08, 2.06, 2.71 kb) and *E. coli* 39R861 (plasmids of 154, 66.2, 37.6, 7.4 kb) (Macrina *et al.*, 1978; Threlfall *et al.*, 1986). Plasmid extraction and molecular size determinations were done in triplicate. Strains were grouped according to their plasmid profiles.

PCR and sequence analysis. PCR was performed to detect and sequence the virulence plasmid markers *seq51* and *seq25* (Lee *et al.*, 2005), the conjugative plasmid markers *traD*, *traI* and *traII*, as well as the whole gene *vep07*, using the primers listed in Table 2. The reaction mixtures (50 µl) for PCR contained 1.2 µl of each primer (10 mM), 0.3 µl *Taq* DNA polymerase (GoTaq, Promega; 5 U µl⁻¹), 10 µl 5 × *Taq* reaction buffer (Promega, GoTaq Green), 3.0 µl 50 mM MgCl₂, 0.5 µl dNTP mix (Promega, 10 mM of each) and 2 µl template DNA (50 ng µl⁻¹). The PCR was performed in a Techne thermocycler (TC-412). The reaction started with 10 min denaturation at 94 °C, followed by 45 cycles of 40 s denaturation at 94 °C, 45 s annealing at the primer-specific temperature shown in Table 2 and 45 s extension

at 72 °C (90 s for amplification of the *vep07* gene); an additional extension at 72 °C for 10 min completed the reaction. A negative control (no template DNA) and a positive control (purified DNA of the serovar E strain CECT 4604) were included in each PCR batch. The amplified products were separated by electrophoresis at 100 V for 30 min on a 2 % (w/v) agarose (Low EEO, Pronadisa) gel in TAE buffer (Real) and were visualized by staining with ethidium bromide.

PCR products were sequenced with the ABI 3730 (Applied Biosystems) and the BigDye Terminator v3.1 Cycle Sequencing kit protocol (Applied Biosystems). Oligonucleotides were purchased from Invitrogen. The resulting sequences were deposited in GenBank (accession nos FJ000003–FJ000061). A multiple sequence alignment was performed using AlignX (Invitrogen) and the evolutionary history for each gene, or gene fragment, was inferred using the neighbour-joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Poisson correction method (Zuckerkanndl & Pauling, 1965), expressed as the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons only. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

Southern hybridization. To localize the marker sequences in the plasmids, Southern hybridization was performed using PCR-labelled amplified products as probes, which were generated by adding 0.15 µl of 10 mM digoxigenin-11-dUTP (Roche) to the PCR mixture. Plasmid DNA was transferred to the membranes (Zeta-probe blotting membrane, Bio-Rad) with a Vacuum Blotter model 785 (Bio-Rad) following the manufacturer's instructions. Hybridization was performed using the DIG Easy Hyb Granules kit (Roche) according to the manufacturer's instructions. The hybridization solution was removed and membranes were washed 2 × 5 min in 2 × SSC containing 0.1 % SDS at room temperature, 2 × 15 min in 0.5 × SSC containing 0.1 % SDS at 65 °C, and 2 × 15 min in 0.1 × SSC containing 0.1 % SDS at 68 °C. Anti-digoxigenin-AP, Fab fragments and Blocking reagent (Roche) were used for development, following the manufacturer's instructions. Membranes were incubated in detection buffer (100 mM Tris/HCl, 100 mM NaCl, pH 9.5; MgCl₂ to 50 mM) for 5 min followed by incubation in coloured buffer (detection buffer + NBT-BCIP, 45 µl of 75 mg NBT ml⁻¹ in dimethylformamide 70 %, 33 µl of 50 mg BCIP ml⁻¹ in dimethylformamide 100 % and 10 ml detection buffer) without light at room temperature for 12–24 h. Finally, membranes were washed twice in distilled water for 2 min, dried and photographed. Plasmid extraction of *E. coli* 39R861, labelled with the Dig-high prime kit (Roche) according to the manufacturer's instructions, was used as a reference to distinguish the positive *V. vulnificus* plasmids for each probe.

Eel virulence. Strains tested for virulence are marked in Table 1. Juvenile European elvers (mean weight 10 g) were maintained in aquaria containing 9 l water [0.5 % (w/v) salinity] at 25 °C. A bacterial dose of 10⁶ c.f.u. per animal was intraperitoneally injected in groups of six animals and mortalities were recorded daily for a 7 day period. A strain was considered virulent if mortality exceeded 50 % (Amaro *et al.*, 1995). Mortalities were only considered to be caused by *V. vulnificus* if the inoculated bacterium was recovered as a pure culture from internal organs. Two groups of animals were challenged with saline solution (0.9 % NaCl, pH 7.0) and included as negative controls. For each *V. vulnificus* strain tested, the LD₅₀ was calculated according to Reed & Muench (1938).

Ribotyping. Representative isolates of each plasmid profile were genetically analysed by automated ribotyping with a Riboprinter (Qualicon). Ribotyping was performed under the conditions recommended by the manufacturer, except that *EcoRI* was replaced by *HindIII* (Roche) (400 U µl⁻¹ in standardized reagents in 1.5 ml tubes). Each isolate was ribotyped twice.

Table 1. Plasmid profile, biotype, serovar and source of the *V. vulnificus* strains used in this study

Strain	Biotype/serovar/ virulence for eels*	Origin	Country of isolation	Plasmid profile†
ATCC 33816, CECT 5164, CECT 5168, CECT 5169, CECT 529 ^T	Bt1/NT/–	Human blood	USA	0 (0)
CS9133	Bt1/NT/–	Human blood	South Korea	0 (0)
CECT 5167, N87	Bt1/NT/–	Human blood	Japan	0 (0)
V4	Bt1/NT/–	Human blood	Australia	0 (0)
CECT 5166	Bt1/NT/–	Wound infection	USA	0 (0)
CECT 4867	Bt1/NT/–	Diseased eel	Sweden	0 (0)
CECT 5165	Bt1/NT/–	Seawater	USA	0 (0)
CG100, CG106	Bt1/NT/–	Oyster	Taiwan	0 (0)
CG110, CG111	Bt1/NT/–	Seawater	Taiwan	0 (0)
E4, JE	Bt1/NT/–	Seafood	USA	0 (0)
L49	Bt1/NT/–	Brackish water	Japan	0 (0)
VV 1003, VV352	Bt1/NT/–	Environmental	USA	0 (0)
KH03	Bt1/NT/–	Human blood	Japan	1 (8)
Riu-1	Bt1/NT/–	Seawater	Spain	2 (20)
VV 425	Bt1/NT/–	Environmental	USA	3 (30)
CG118	Bt1/NT/–	Seawater	Taiwan	3 (30)
94385	Bt1/NT/–	Leg wound	Spain	4 (48)
94-9-118	Bt1/NT/–	Expectoration from lungs	Denmark	4 (48)
94-9-130	Bt1/NT/–	Water	Denmark	4 (48)
A2, A4, A5, A6, A7	Bt1/NT/–	Eel tank water	Spain	4 (48)
CECT 4606	Bt1/NT/–	Healthy eel	Spain	4 (48)
PD-1, PD-12, PD-2-66#, PD-3, PD-5, V1	Bt1/NT/–	Eel tank water	Spain	4 (48)
YJ106	Bt1/NT/–	Human blood	Taiwan	4 (48)
YN03	Bt1/NT/–	Human blood	Japan	4 (48)
CECT 4608	Bt1/NT/–	Eel farm water	Spain	5 (20, 7)
MLT404	Bt1/NT/–	Environmental	USA	6 (20, 9)
MLT 364#	Bt1/NT/–	Environmental	USA	7 (33, 3)
94-9-119	Bt1/NT/–	Human wound	Denmark	8 (48, 4)
MLT 406	Bt1/NT/–	Environmental	USA	9 (48, 24)
MLT 362	Bt1/NT/–	Environmental	USA	10 (48, 35)
Riu-3	Bt1/NT/–	Seawater	Spain	11 (49, 36)
CECT 5768	Bt2/SerA/ +	Diseased eel	Spain	12 (70)
21A	Bt2/SerA/ +	Diseased eel	Spain	13 (70, 52)
A13#, A21, A22, CECT 5689, CECT 5769	Bt2/SerA/ +	Diseased eel	Spain	14 (70, 55)
A15, A16, A17, A18, CECT 5198, CECT 5343	Bt2/SerA/ +	Diseased eel	Spain	15 (70, 52, 30)
22, 26, 27, 21B, 4/7/17, 4/7/19	Bt2/SerA/ +	Diseased eel	Denmark	15 (70, 52, 30)
A10, A11, A14	Bt2/SerA/ +	Diseased eel	Spain	16 (70, 55, 21)
94-8-112	Bt2/SerE/ +	Human wound	Denmark	17 (68)
CECT 4864, CECT 4999	Bt2/SerE/ +	Diseased eel	Spain	17 (68)
94-9-123	Bt2/SerE/ +	Seawater	Denmark	18 (68, 30)
CECT 4605, CECT 4607	Bt2/SerE/ +	Diseased eel	Spain	18 (68, 30)
CECT 4604	Bt2/SerE/ +	Diseased eel	Spain	19 (68, 52)
CECT 898	Bt2/SerE/ +	Diseased eel	Japan	20 (68, 53)
UE516	Bt2/SerE/ +	Diseased Japanese eel	Taiwan	20 (68, 53)
CIP8190	Bt2/SerE/ +	Human blood	France	21 (68, 56)
CECT 4866	Bt2/SerE/ +	Human blood	Australia	21 (68, 56)
CCUG 38521	Bt2/SerE/ +	Human wound	Sweden	21 (68, 56)
CECT 4602, CECT 4603	Bt2/SerE/ +	Diseased eel	Spain	21 (68, 56)
Ö122	Bt2/SerE/ +	Diseased eel	Sweden	21 (68, 56)
PD-2-47, PD-2-50, PD-2-51#, PD-2-55, PD-2-56, CECT 5763	Bt2/SerE/ +	Eel tank water	Spain	21 (68, 56)
C1, CECT 5762	Bt2/SerE/ +	Healthy eel	Spain	21 (68, 56)
Riu-2	Bt2/SerE/ +	Seawater	Spain	21 (68, 56)
CECT 4601	Bt2/SerE/ +	Diseased eel	Spain	22 (68, 56, 10)
CECT 897, CECT 4174	Bt2/SerE/ +	Diseased eel	Japan	23 (70, 56, 10)

Table 1. cont.

Strain	Biotype/serovar/ virulence for eels*	Origin	Country of isolation	Plasmid profile†
CECT 4865	Bt2/SerE/ +	Diseased shrimp	Taiwan	24 (68, 56, 30)
CECT 4868	Bt2/SerE/ +	Diseased eel	Norway	24 (68, 56, 30)
90-2-11	Bt2/SerE/ +	Diseased eel	Denmark	24 (68, 56, 30)
CECT 4862	Bt2/SerE/ +	Diseased eel	Japan	25 (70, 52, 34, 20)
95-8-161, 95-8-162	Bt2/SerI/ +	Diseased eel	Denmark	26 (70, 55, 5)
CECT 4869#‡	Bt2/SerI/ –	Diseased eel	Belgium	27 (70, 54, 34, 5)
95-8-6, 95-8-7	Bt2/SerI/ +	Diseased eel	Denmark	27 (70, 54, 34, 5)
97, 162, 11028, vv12, vv32	Bt3/SerO/ –	Diseased human	Israel	28 (48, 5)

*Bt, biotype; NT, non-typable; SerE, serovar E; SerA, serovar A; SerI, serovar I; SerO, serovar O. Data on serology and virulence from Lee *et al.* (2005), Fouz *et al.* (2007) and from this work (strains marked # in column 1).

†The molecular mass of the plasmids is indicated in parentheses.

‡Spontaneous mutant in *vep07*.

RESULTS

Plasmid profiles

A total of 28 plasmid profiles were found in the 112 analysed strains (Table 1). Biotype 1 strains produced 12 different profiles, biotype 2 produced 14, and biotype 3 possessed a unique profile. Of the biotype 1 strains, 42.8 % lacked plasmids (profile 1), and an identical percentage possessed a plasmid similar in size to the conjugative plasmid pYJ106 (47–48 kb). A plasmid of around 30 kb was recorded in 8.6 % of biotype 1 strains, while a 20 kb

plasmid was found in 6.8 %, and a plasmid of between 3 and 8 kb in 8.6 % of these strains (Table 1). All biotype 2 strains possessed a plasmid of molecular size similar to the virulence plasmid described in strains CECT 4999 and CECT 4602 (68–70 kb) (Lee *et al.*, 2008) and 88 % possessed a second plasmid of a molecular size similar to the conjugative plasmid p4602-1 (52–56 kb). In addition, 22.4 % of biotype 2 strains possessed a plasmid of around 30 kb, 6.9 % had a plasmid of 20 kb, and 13.8 % a plasmid of between 5 and 10 kb (Table 1). The profile generated by biotype 3 strains corresponded to a plasmid of 48 kb and a second plasmid of 5 kb (Table 1).

Table 2. Oligonucleotides used in this study to analyse plasmid diversity

Primer	Sequence	Annealing temp. (°C)	Size (bp)	Use
Seq25F	TGCTCAAAGCCATACTCTCT	52	411	Marker for virulence plasmid
Seq25R	GCCAAGTGCTAATCCATCC			
Seq51F	AGAGATGGAAGAAACAGGCG	52	344	Marker for virulence plasmid
Seq51R	GGACAGATACAAGGGCAAATGG			
TraIF	CTGAGCACCACCAAGGC	52	652	Marker for conjugative plasmid
TraIR	CGTGTACGCTGTTGTCCCT			
TraI2F	GAAGCGAGACGATCACAA	54	782	Marker for virulence/conjugative plasmid
TraI2R	AGTGGTCAATCGCTCTGCTG	53	619	Marker for virulence/conjugative plasmid
TraDF	CACCAGTAAGTTCTTCGACC			
TraDR	CTCGTCCATAATCACCCAGA	50.2	1494	Primers to sequence the <i>vep07</i> gene
Vep07F	TCTCCTCTAGGCGATTACCG			
Vep07R	CACCCGAATTAATCAGC			

Table 3. Changes in *vep07* in the spontaneous mutant CECT 4869

	Changes in nt			Amino acid sequence from residue 80
	293–294	268	263	
Seq51 CECT 4869	AA	*	A	...DQELIRTDIRQMEILEAANLLALMESY†
Seq51 GenBank AY691409	TT	C	G	...DQELIRTDTRANGNFGSSISVSSNGKLLVVSAAQNTPSEGKK...

*Point deletion.

†Stop codon.

Plasmid analysis

Identification of the virulence plasmid for eels. First, a PCR was conducted with all the strains to confirm that the virulence plasmid was present in all biotype 2 strains and absent in all biotype 1 and 3 strains. A PCR product of the predicted size was found by PCR with primers Seq25FR and Seq51FR in all biotype 2 strains (profiles 13–27) with the exception of the serovar I strain CECT 4869, which was positive for *seq25* but negative for *seq51*. Additional PCRs were performed with primer Seq51FR for this strain, decreasing the annealing temperature to 40 °C, and a PCR product of the correct size was obtained. This product was sequenced and compared with the database (Lee *et al.*, 2005) and the differences detected are shown in Table 3. *seq51* is

located within *vep07*, a virulence gene (GenBank accession no. FJ000051). The whole gene was sequenced in strain CECT 4869 and in a selection of biotype 2 strains (one for each plasmid profile group). The gene showed 100 % homology for the majority of the strains with the exception of four isolates: CECT 4601 and 94-9-112, which had a non-coding change at position 120 (G instead of T), and CECT 5769 and 95-8-161, which had one amino acid substitution at position 171 (arginine for glycine). The distance tree for the *vep07* protein is shown in Fig. 1. All the strains with homology between 99.7 % and 100 % for *vep07* were virulent for eels (percentage mortalities after injection of 10⁶ c.f.u. per fish greater than 50 %) (Table 1). The strain CECT 4869, which produces a different protein, was avirulent for eels by

Fig. 1. Neighbour-joining dendrogram for the *V. vulnificus* isolates based on variation in a fragment of TraD (A) and TraI (B) and the whole protein Vep07 (C). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The bar indicates the relative sequence divergence.

Fig. 2. Southern hybridization with probes Seq25 (A) and Seq51 (B). Lanes: 1, pre-stained 39R861 (see Methods); 2, CECT 5198; 3, CECT 5768; 4, CECT 5769; 5, A14; 6, 21A; 7, 94-8-112; 8, 94-9-123; 9, CECT 4602; 10, CECT 898; 11, CECT 4604; 12, 90-2-11; 13, CECT 4601; 14, CECT 897; 15, CECT 4862; 16, 95-8-161; 17, 95-8-6; 18, YJ016; 19, CG100; 20, 11028; 21, VV12.

intraperitoneal injection since none of the fish died after injection of 10^6 c.f.u. per fish 7 days post-infection (Table 1).

The plasmid carrying *seq25* and *seq51* was identified by hybridization with probes derived from the amplified products. In all cases, only the plasmid of the highest molecular mass (68–70 kb) hybridized with the probes (Fig. 2). None of the biotype 1 and biotype 3 plasmids hybridized with the probes from eel virulence markers and none of the strains were virulent for eels (Table 1).

Identification of the conjugative plasmid. The primer pairs TraDR/F, TraIR/F and TraI2R/F were designed to detect the *traD*, *traI* and *traI'* genes. The *traI'* gene is a non-functioning truncated pseudogene present in virulence plasmids pR99 and pC4602-1 (Lee *et al.*, 2008).

Fig. 3 shows the regions in which the primers annealed and their derived probes. Three PCR products of the predicted size were obtained in strains with the plasmid profiles 4, 8–11, 13–16 and 19–28, regardless of biotype or serovar, whereas a unique PCR product of the predicted size was obtained with primers TraI2F/R in strains corresponding to plasmid profiles 12 and 17 (data not shown). Based on the primer design, only plasmids that hybridized with all the probes could be considered as conjugative. Plasmids of 48–49 kb present in biotype 1 strains (profiles 5 and 9–12), the biotype 3 strain (profile 28), as well as the plasmids of 52–56 kb present in biotype 2 strains (profiles 14–17 and 20–27) hybridized with all three probes and were identified as putative conjugative plasmids (Fig. 3). Biotype 2 plasmids of molecular size 68–70 kb hybridized with the TraI2 probe, but not with the TraI probe (Fig. 3).

The presence of *traD* and *traI* was further confirmed by sequence analysis of the PCR products of selected positive strains from each plasmid profile group. In Fig. 1 the distance tree for a fragment of the proteins TraD and TraI is shown. The similarity of these protein sequences with

that of pYJ016 was higher than 98.2 % for TraD and 98.3 % for TraI.

Ribotyping

Representative strains of the different plasmid profiles were analysed by ribotyping; the results are shown in Fig. 4. With the exception of biotype 3 strains, no correspondence between ribopattern and plasmid profile was observed:

Fig. 3. Southern hybridization results for *traD*, *traI* and *traI2*. (A) Diagram of the probe design to detect plasmids with truncated *traI*: a) conjugative plasmid; b) virulence plasmid. (B) Southern hybridizations of pre-stained 39R861 (lane 1), YJ016 (2), 11028 (3), CECT 4604 (4), A14 (5) and 95-8-162 (6) with probe TraD (B), probe TraI (C) and probe TraI2 (D).

Fig. 4. Ribotypes obtained with *Hind*III for representative strains of each plasmid profile of *V. vulnificus*.

different ribopatterns were shown by strains belonging to the same plasmid profile group and, conversely, a common ribopattern was generated by strains belonging to different plasmid profile groups (Fig. 4).

DISCUSSION

The species *V. vulnificus* is one of the vibrio species whose genome has been sequenced. The two sequenced strains show a high degree of chromosomal similarity, which does not reflect the high degree of variability and plasticity displayed by a species able to colonize hosts as different as humans and fish and act as both pathogen and commensal microbiota. In contrast to the homology of their chromosomes, the two strains differ in their plasmid content, which is of particular interest given that previous studies have reported a general lack of extrachromosomal elements within this species. The genetic homogeneity of these two strains is probably related to the characteristics of the isolates chosen for this study (both biotype 1 human isolates were recovered from human blood from two proximal countries in South-east Asia: Taiwan and Korea); greater chromosomal variation is to be expected by sequencing strains from more diverse sources. Despite this fact, our results suggest that plasmids could play a role in generating diversity within the species.

Our work reinforces the putative role of plasmids in the evolution and environmental adaptation of *V. vulnificus*. First, most of the strains analysed, especially those belonging to biotypes 2 and 3, harboured plasmids and almost 30 different plasmid profiles were found in a total of 112 strains. These plasmid profiles were biotype- or serovar-specific, since no profile was shared by strains of different biotypes or by strains of different serovars. In contrast, no correlation was found between the origin of a strain and its plasmid profile, since the same profile was shared by clinical and environmental strains, and by strains isolated from Asia, Europe or America. Almost half of the biotype 1 strains lacked plasmids, partially confirming previous work performed with clinical and environmental biotype 1 strains, which reported a general lack of extrachromosomal elements within the species (Davidson & Oliver, 1986). Most of the remaining biotype 1 strains harboured a putative conjugative plasmid of a molecular size similar to that of pYJ016 (Chen *et al.*, 2003). In fact, the sequence analysis of a fragment of *traD* and *traI* from this putative conjugative plasmid revealed that the gene fragments are almost identical to those of pYJ016, and thus all these plasmids probably belong to the same family of F-plasmids.

In relation to biotypes 2 and 3, 100 % of the strains carried plasmids. Interestingly, all biotype 2 strains, regardless of serovar, carried the virulence plasmid, a plasmid recently described in two serovar E isolates (Lee *et al.*, 2008). These two plasmids (pC4602-2 and pR99) have been sequenced and display 92 % homology, the main difference between them being a *mazEF* operon (coding for a toxin/antitoxin system) present in pR99 and absent in pC4602-2 (Lee *et al.*, 2008). Half the plasmid ORFs were annotated as hypothetical and, of the remainder, an *rtx* gene cluster and the ORF *vep07*, which contains *seq51* and a part of *seq25*, were highlighted. Mutations in this gene produced a significant

reduction in virulence for eels and survival in eel blood, which suggests that this gene codes for a novel virulence factor (Lee *et al.*, 2008).

Our results confirm the importance of *vep07* for virulence, since (i) mutations that produced a change in this gene resulted in a loss of eel virulence and (ii) the homology of the protein inferred by comparison of sequences of *vep07* in different strains from different origins and serovars was between 99.7 and 100 %.

Interestingly, almost 90 % of biotype 2 strains also carried a putative conjugative plasmid, similar in size to the conjugative plasmid pC4602-1. Plasmid pC4602-1 displays a high sequence similarity to pYJ016, in terms of both DNA sequence and gene organization (Lee *et al.*, 2008). Sequence analysis of a fragment of the *traD* and *traI* genes performed in our study confirms that biotype 2 plasmids are of the same family of F-plasmids as pYJ016. These plasmids have been demonstrated to be essential for transmission of the virulence plasmid by means of conjugation experiments (Lee *et al.*, 2008). In those experiments the virulence plasmid was transmitted with a frequency of 3.9 % and almost a third of the transconjugants carried only the virulence plasmid. In our study, 6.9 % of the biotype 2 strains possessed only the virulence plasmid, which is in accordance with the study of Lee *et al.* (2008), and supports the hypothesis that the conjugative plasmid plays an essential role in the natural transmission of the virulence plasmid.

In relation to biotype 3, all strains showed an identical plasmid profile, and furthermore, all contained a putative conjugative plasmid identical in size to pYJ016. The sequence analysis of *traD* and *traI* revealed that the biotype 3 conjugative plasmid probably belongs to the same family of F-plasmids as that of YJ016.

In addition, we examined the relationship between chromosomal and plasmid typing by comparing ribopatterns and plasmid profiles. No correspondence was found between biotype 1 and 2 strains, since the same plasmid profile was presented by strains with different ribopatterns and, conversely, different plasmid profiles were associated with the same ribopattern. In the case of biotype 3 strains, the strains showed the same ribopattern and the same plasmid profile, which reinforces the hypothesis that this biotype constitutes a clonal complex formed by genetically identical strains (Bisharat *et al.*, 2007b). Previous ribotyping studies in *V. vulnificus* have suggested that this technique could be used as an epidemiological tool to distinguish clones within the species. Our results demonstrate that this technique should be used in combination with plasmid profile determination, which allows strains of the same ribopattern to be distinguished. Conversely, ribotyping enables strains of the same plasmid profile to be distinguished.

In summary, *V. vulnificus* possesses plasmids. The virulence plasmid is limited to biotype 2 strains and is

absolutely essential for eel virulence, whereas conjugative plasmids of the same family are widely distributed among the three biotypes and could have contributed to the transmission of the virulence plasmid. All these plasmids probably play an important role in environmental adaptation and plasticity of the species.

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