Phenotypic and genotypic characterization of a new fish-virulent *Vibrio vulnificus* serovar that lacks potential to infect humans

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Vibrio vulnificus is a bacterial species that is virulent for humans and fish. Human isolates are classified into biotypes 1 and 3 (BT1 and BT3) and fish isolates into biotype 2 (BT2). However, a few human infections caused by BT2 isolates have been reported worldwide (zoonosis). These BT2 human isolates belong to serovar E (SerE), which is also present in diseased fish. The aim of the present work was to characterize a new BT2 serovar [serovar A (SerA)], which emerged in the European fish-farming industry in 2000, by means of phenotypic, serological and genetic [plasmid profiling, ribotyping and random amplified polymorphic DNA (RAPD)] methodologies. The results confirmed that SerA constitutes a homogeneous O-serogroup within the species that shares plasmidic information with SerE. Like SerE, this new serogroup was resistant to fresh fish serum, as well as being highly virulent for fish. In contrast, it was sensitive to human serum and avirulent for mice, even after pretreatment with iron. The two serovars presented different biochemical profiles as well as specific patterns by ribotyping and RAPD analysis. In conclusion, SerA seems to constitute a different clonal group that has recently emerged within the species *V. vulnificus*, with pathogenic potential for fish but not for humans.

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INTRODUCTION

Vibrio vulnificus is an aquatic bacterium from warm and tropical ecosystems that produces diseases in humans and aquatic animals (Tison *et al.*, 1982; Oliver, 1989; Amaro & Biosca, 1996). Human vibriosis occurs after eating contaminated seafood or exposing open wounds to seawater (Veenstra *et al.*, 1992; Strom & Paranjpye, 2000), and fish vibriosis after immersion in contaminated water or contact with diseased animals or carriers (Amaro *et al.*, 1995; Marco-Noales *et al.*, 2001; Valiente & Amaro, 2006).

The species has been subdivided into three biotypes on the basis of differences in biochemical properties, such as indole production and cellobiose fermentation, as well as serological and genetic traits and host range (Tison *et al.*, 1982; Bisharat *et al.*, 1999, 2007). Biotypes 1 (BT1) and 3 (BT3) are considered human pathogens and biotype 2 (BT2) pathogens of aquatic animals (Tison *et al.*, 1982; Song *et al.*, 1990; Biosca *et al.*, 1996, 1997; Bisharat *et al.*, 1999; Fouz *et al.*, 2002, 2006a; Fouz & Amaro, 2003) in spite of the fact that several human cases of wound infection and sepsis due to BT2 have occurred worldwide

(Veenstra *et al.*, 1992; Amaro & Biosca, 1996; Dalsgaard *et al.*, 1996). BT2 was first isolated in Japan in 1976 (Muroga *et al.*, 1976) and was described in 1982 by Tison *et al.* (1982). The first cases in Europe were recorded in 1989 in Spanish eel farms (Biosca *et al.*, 1991). Later, the disease spread to other countries such as Sweden, The Netherlands and Denmark (Høi *et al.*, 1998; Dalsgaard *et al.*, 1999), always affecting eels cultured in brackish water.

BT2 is heterogeneous and can be subdivided into different serovars (Martin & Siebeling, 1991; Biosca *et al.*, 1996, 1997; Høi *et al.*, 1998; Fouz & Amaro, 2003). The first described, serovar E (SerE) (equivalent to serovar O4 according to the serotyping system of Martin & Siebeling, 1991), is clearly related to both highly virulent epizootics and human infections (Veenstra *et al.*, 1992; Amaro & Biosca, 1996; Dalsgaard *et al.*, 1996). This serovar seems to be genetically homogeneous regardless of origin, either human or fish (Gutacker *et al.*, 2003). The last serovar described, serovar A (SerA), emerged in Southern Europe in 2000 (Fouz & Amaro, 2003), affecting eels cultured in fresh water that had been vaccinated against SerE (Fouz *et al.*, 2001). SerA spread to the Danish eel farming industry in the summer of 2004, also affecting eels cultured in fresh water (Fouz *et al.*, 2006a). Two additional serovars, O3 and O3/O4 (SerO3 and SerO3/O4), were only isolated once from diseased eels as secondary pathogens in Denmark in the mid-1990s (Høi *et al.*, 1998). They were

Abbreviations: BT, biotype; ECP, extracellular product; GR, growth rate; Hb, haemoglobin; RAPD, random amplified polymorphic DNA; Ser, serovar; SS, sterile saline; UPGMA, unweighted pair group method with arithmetic means.

avirulent for eels by waterborne infection (Fouz *et al.*, 2001). For this reason, their inclusion in BT2 is controversial.

To gain insight into the virulence factors that are essential for eel pathogenicity, genomes of selected strains belonging to the different biotypes, both virulent and avirulent for eels, were compared by suppression subtractive hybridization (Lee *et al.*, 2005). This study demonstrated that all virulent BT2 strains analysed, irrespective of the serovar, shared plasmid-borne genetic sequences that could be considered as virulence markers for fish (Amaro *et al.*, 2006).

The aim of the present work was to study the new emergent SerA in depth and to compare it with SerE. To this end, SerA isolates from different geographical origins were serologically, biochemically and genetically characterized, using reference strains of BT1, BT2 and BT3. Plasmid profiling, ribotyping and random amplified polymorphic DNA (RAPD) fingerprinting were chosen from among the different techniques available for epidemiological analysis, because they have been used by other authors to study

intraspecific differentiation of *V. vulnificus* (Aznar *et al.*, 1993; Biosca *et al.*, 1997; Arias *et al.*, 1997, 1998; Høi *et al.*, 1997; Gutacker *et al.*, 2003). Finally, the virulence for eels and mice (as a model for pathogenic potential for humans) of SerA isolates was also evaluated.

METHODS

Bacterial strains and growth conditions. A total of 30 strains of *V. vulnificus* SerA of different origins, as well as control strains of BT1, BT2 SerE and BT3, were used for this study (Table 1). SerA strains were isolated as pure cultures of opaque colonies (capsulated) from the internal organs, jaw or external ulcers of diseased eels (Fouz & Amaro, 2003; Fouz *et al.*, 2006a). The isolates were identified by colony hybridization using a *V. vulnificus*-specific alkaline-phosphatase-labelled DNA probe (DNA Technology) directed against the haemolysin/cytolysin gene *hlyA* (Wright *et al.*, 1993) and by slide-agglutination with specific anti-SerA serum (Fouz & Amaro, 2003; Fouz *et al.*, 2006a). Bacterial strains were routinely grown on Tryptic Soy Agar (Oxoid) or Tryptic Soy Broth (Oxoid) supplemented with 0.5% (w/v) NaCl (TSA-1 or TSB-1) at 28 °C for 24 h. The strains were maintained both as lyophilized stocks and as frozen stocks at –80 °C in marine broth (Difco) plus 20% (v/v) glycerol.

Table 1. Origin, biotype, serovar, biochemical characteristics and plasmid profile of *V. vulnificus* strains

Strain*	Source†	Country, year‡	API 20E profile§	Biotype	Serovar	Plasmid profile¶
A1 (CECT 5198), A1.2, A1.3, A1.4, A1.5#	IO, diseased eel	Spain, 2000 (IF1)	5146105	BT2	A	1 (≈75, 50, 30)
A3 (CECT 5343), A3.2, A3.3, A3.4, A3.5#	IO, diseased eel	Spain, 2001 (IF1)	5146105	BT2	A	1
A8 (CECT5768)	IO, diseased eel	Spain, 2001 (WE)	5146105	BT2	A	2 (≈75)
A9 (CECT5769)	Ulcer, diseased eel	Spain, 2002 (IF1)	5146105	BT2	A	3 (≈75, 60–65)
A10, A11, A14	IO, diseased eel	Spain, 2002 (IF1)	5146105	BT2	A	4 (≈75, 60–65, 20)
A12 (CECT5689), A13	IO, diseased eel	Spain, 2002 (IF1)	5146105	BT2	A	3
A15, A16**	IO, diseased eel	Spain, 2003 (WE)	5146105	BT2	A	1
A17, A18	IO, diseased eel	Spain, 2005 (IF2)	5146105	BT2	A	1
A19, A20	IO, diseased eel	Spain, 2005 (IF1)	5146105	BT2	A	1
17 (CECT 7029), 19 (CECT 7030), 21 B, 22, 26, 27#	Jaw or IO, diseased eel	Denmark, 2004 (IF3)	51(3)46105	BT2	A	1
21A#	IO, diseased eel	Denmark, 2004 (IF3)	5146105	BT2	A	5 (≈75, 50)
CECT 4604	IO, diseased eel	Spain, 1990	5006005	BT2	E	6 (69, 57)
ATCC 27562 ^T	Human blood	United States	5146105	BT1	Non-SerE, non-SerA	–
VV12	Human	Israel	4146005	BT3	Non-SerE, non-SerA	–

*SerA isolates characterized in previous studies are marked with #CECT, Spanish Type Culture Collection; **non-motile strains; ATCC, American Type Culture Collection. T, type strain.

†IO, internal organ.

‡Isolates were recovered from diseased eels in outbreaks occurring in intensive farms (IF1, IF2 and IF3) or from diseased wild eels (WE).

§Identification according to the API database: 5146105, *V. vulnificus* (99.7%); 5006005, *V. vulnificus* (54.4%) or *Burkholderia cepacia* (45.3%).

||Biotype 2 serovars were determined by slide-agglutination test with polyclonal antisera against serovars A and E.

¶1–5, Profile of SerA strains on the basis of their plasmid content. 6, Profile of the SerE strain used as control. Molecular mass of plasmid bands is expressed in kilobases (kb) in parentheses. –, without plasmids.

Biochemical characterization. For biochemical characterization, all strains were seeded in API 20E strips (BioMérieux) and the profiles were compared with the API database (Apilab Plus, version 3.3.3; BioMérieux) (Amaro *et al.*, 1992b; Sanjuán & Amaro, 2004). Motility was assayed on TSA-1 motility plates containing 0.3% (w/v) Bactoagar (Difco) (Gardel & Mekalanos, 1996). Antibiotic susceptibility was checked by the disc diffusion method on Mueller–Hinton agar (Difco) supplemented with 1% (w/v) NaCl (Amaro *et al.*, 1992b) using tetracycline (20 µg per disc), flumequine (30 µg per disc), sulfamethoxazole/trimethoprim (25 µg per disc) and oxolinic acid (2 µg per disc) (Oxoid). Inhibition zones were read following incubation at 28 °C overnight.

Serological characterization. *V. vulnificus* isolates were serologically characterized by slide-agglutination with rabbit polyclonal antisera according to the previously described procedure (Amaro *et al.*, 1992b). Antisera were prepared by intravenous injection of New Zealand rabbits with formalin-killed cells from strains of SerE (CECT 4604), SerA (CECT 5198), BT1 (ATCC 27562) and BT3 (VV12) according to the procedure described by Sorensen & Larsen (1986). Antisera were also prepared against serovars O3 (strain 95-8-6) and O3/O4 (strain 95-8-161), occasionally involved in eel infections as secondary pathogens (Høi *et al.*, 1998). Fresh and heated (100 °C for 2 h) whole-cell suspensions containing 10^8 c.f.u. ml⁻¹ of each strain were used as antigens (Amaro *et al.*, 1992b). A distinct and immediate agglutination was registered as positive. LPS extracts of all strains were prepared from both whole-cell lysates and extracellular products (ECPs) obtained by the cellophane technique (Liu, 1957) basically according to the method of Hitchcock & Brown (1983). LPS samples from all strains were subjected to SDS-PAGE (Laemmli, 1970). LPS components fractionated by SDS-PAGE were analysed by both silver nitrate staining and immunostaining as previously described (Amaro *et al.*, 1992a). In the latter case, LPS were transferred from the polyacrylamide gel to nitrocellulose sheets (0.45 µm; Bio-Rad) basically by the procedure of Towbin *et al.* (1979). Blotting was done at 200 mA for 2 h in Tris-glycine-methanol transfer buffer [25 mM Tris, 192 mM glycine, pH 8.3, and 20% (v/v) methanol]. LPS bands were visualized by immunostaining with serovar-specific polyclonal antibodies (dilutions 1:200–1:1000) according to previously described procedures (Amaro *et al.*, 1992a).

Plasmid analysis and PCR. Extraction of plasmid DNA was performed by the TENS method (Zhou *et al.*, 1990) with slight modifications. Bacterial cells were grown in Luria Broth (Difco) for 6 h and frozen before starting the protocol, which included at least two steps of treatment with phenol. After DNA resuspension at room temperature, samples were subjected to electrophoresis on 0.7% (w/v) agarose gels (Roche, molecular grade) for 3 h at 50 V. Molecular sizes of SerA plasmids were estimated using several reference plasmids from *V. vulnificus* SerE strain CECT 4999 (69 kb) and CECT 4604 (69, 57 kb), *Escherichia 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).

The presence of the identified BT2-specific plasmid-borne sequence 51 (seq51) (Lee *et al.*, 2005; Amaro *et al.*, 2006) in plasmid samples was tested using PCR (Lee *et al.*, 2005). The accession number for this sequence seq51 in GenBank is AY691409 (Lee *et al.*, 2005). The reaction mixture (50 µl) contained 200 ng DNA, each deoxynucleoside triphosphate at a concentration of 0.2 µM, 75 mM MgCl₂, each primer at a concentration of 10 µM (VF51, 5'-GGACA-GATACAAGGGCAAATGG-3', and VR51, 5'-AGAGATGGAAGAA-ACAGGCG-3') and 2.5 U *Taq* polymerase (Amersham Biosciences) in PCR buffer (Amersham Biosciences). The reaction started with 5 min of denaturation at 94 °C, which was followed by 25 cycles of 30 s of denaturation at 94 °C, 1 min of annealing at 55–65 °C and 1 min of extension at 72 °C. 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 SerE strain CECT 4604) were included in each PCR batch. The amplified products were separated by electrophoresis on 1.8% (w/v) agarose gels and were visualized by staining with ethidium bromide. Strain CECT 4604 of SerE was used as positive control.

Ribotyping. The SerA isolates were characterized by automated ribotyping with the Riboprinter (Qualicon). Ribotyping was performed under the conditions recommended by the manufacturer (Clermont *et al.*, 2001) except that *Eco*RI was replaced by *Hind*III (Roche) (400 U µl⁻¹ in standardized reagents in 1.5 ml tubes). Each isolate was ribotyped twice.

RAPD-PCR analysis. Chromosomal DNA was extracted by the method outlined by Pitcher *et al.* (1989) and further purified by RNase and proteinase K treatments as described by Sambrook *et al.* (2001). RAPD analysis was performed using the universal primers M13 (5'-GAAACAGCTATGACCATG-3') and T7 (5'-AATACGACTCACTA-TAGG-3'). PCR was conducted in a total volume of 50 µl containing 1 µl universal primer (50 mM), 0.5 µl *Taq* DNA polymerase (Genotag; 5 U µl⁻¹), 5 µl *Taq* reaction buffer, 2.5 µl 100 mM MgCl₂, 5 µl of each deoxynucleoside triphosphate (10 mM) and 5 µl template DNA (50 ng µl⁻¹). Reaction mixtures were subjected to one cycle (MJR thermocycler) of 94 °C for 5 min, followed by 45 cycles of 94 °C for 40 s, 36 °C for 1 min and 72 °C for 1 min. This was followed by a cycle of 72 °C for 10 min. The amplification products were electrophoresed at 100 V for 3 h on a 1.2% (w/v) agarose (Low EEO, Pronadisa) gel in TBE buffer (0.89 mol Tris base l⁻¹; 0.89 mol boric acid l⁻¹; 0.02 mol Na₂EDTA l⁻¹, pH 8.3). Gels were stained with ethidium bromide and photographed under UV light. Gel images were recorded with a video camera (GelPrinter Plus, TDI) and stored as TIFF files. Digitized images were converted, normalized and analysed with the software package Gel Compar version 4.1 (Applied Maths). Similarity among band patterns was calculated with the Pearson product-moment correlation coefficient and dendrograms were constructed by the unweighted pair group method with arithmetic means (UPGMA). A band position tolerance of 1% was allowed to compensate for misalignments of homologous bands due to technical imperfections. The assay was done in duplicate.

Resistance to eel and human sera. The survival in fresh eel and human sera was assayed with stationary-phase bacteria in microtitre plates (Sanjuán & Amaro, 2004). In each well, a volume of 20 µl serum was mixed with 20 µl of a suspension of bacteria (10^4 – 10^5 c.f.u. ml⁻¹) in sterile saline solution [0.9% (w/v) NaCl, pH 7, SS]. The assays were performed in triplicate and samples were obtained at 0, 1, 2 and 4 h incubation at 28 °C (eel serum assay) or 37 °C (human serum assay). Viable counts were determined by drop plating on TSA-1. The final growth rate (GR) was calculated for each strain as the log of the final count divided by the initial count and was coded as follows: 1 (0 < GR ≤ 1), 2 (1 < GR ≤ 2), or 3 (3 < GR).

Virulence assays. Virulence for eels was assayed using juvenile European elvers (mean weight of 10 g). Fish were maintained in aquaria containing 9 l water [0.5% (w/v) salinity] at 25 °C. In order to study the pathogenic potential for humans, the virulence for BALB/c mice (mean weight 20 g) was also assayed. Since the virulence of *V. vulnificus* increases greatly when haemoglobin (Hb) is injected before bacterial infection (Amaro & Biosca, 1996), groups of mice were also pretreated with Hb (1.5–2.15 µg Fe as Hb per g body weight; iron-loaded mice) using the induced peritonitis model (Helms *et al.*, 1984). Bacterial doses ranging from 10^1 to 10^8 c.f.u. per animal were intraperitoneally injected as previously described (Amaro *et al.*, 1992a). Groups of six animals were used for each experiment and mortalities were recorded daily for 7 days. Mortalities were only considered to be caused by *V. vulnificus* if the inoculated bacterium was recovered as pure culture from internal organs. For identification, we used the slide-agglutination test with the corresponding serum. Two groups of

animals were challenged with SS and included as negative controls. For each *V. vulnificus* strain tested, the LD₅₀ was calculated by the procedure of Reed & Muench (1938).

RESULTS

Biochemical characterization

All SerA isolates gave the same API 20E profile as the type strain of the species, which belongs to BT1 (Table 1). This profile differed from the SerE profile in indole production, ornithine decarboxylation and acid production from D-mannitol, tests negative for SerE and positive for SerA. Moreover, SerA isolates differed from BT3 in ONPG activity and acid production from D-mannitol. According to the API database, this profile would correspond to *V. vulnificus* with a probability of 99.7 %. Most of the strains were motile, with the exception of isolates recovered from wild eels in 2003 (Table 1), which were unable to penetrate motility agar. All of them were also sensitive to all the antibiotics tested, as was the SerE isolate used as control in the susceptibility assays.

Serological characterization

All SerA isolates gave a strong positive reaction with the anti-SerA serum and a negative response with the antisera against the type strain (BT1), BT3 and SerE, regardless of the antigen used in the tests, fresh or heated whole-cell suspensions. SerA strains exhibited slight cross-reactions with O3 and O3/O4 antisera when the agglutination tests were carried out with fresh whole cells, which disappeared when heated cells were used.

The LPS of isolates were further analysed by Western blotting with BT2 antisera. LPS of SerA isolates were visualized after immunostaining with anti-SerA serum (Fig. 1) but not after silver staining or after immunostaining with anti-SerE serum (data not shown). Conversely, LPS from the SerE isolate did not react with anti-SerA serum (Fig. 1). All SerA LPS samples, those obtained from both whole-cell lysates and ECPs, exhibited the same pattern, which presented a ladder-like structure typical of smooth LPS (Fig. 1). This band pattern differed from that shown by the SerE strain (Fig. 1). Thus, two highly immunogenic zones of middle and fast mobility were identified in the SerA-LPS band pattern, whereas only one zone of low mobility was identified in the SerE-LPS pattern. Finally, the fast-migrating band corresponding to the lipid A and core region of LPS from SerO3, SerO3/O4 and BT3 strains was stained with anti-SerA serum (Fig. 1). Similarly, the same moiety of LPS of SerA isolates was stained with anti-SerO3, anti-SerO3/O4 and anti-BT3 sera (data not shown).

Plasmid analysis and PCR

All SerA isolates harboured a large plasmid of approximately 75 kb (Fig. 2, Table 1). This plasmid was slightly

Fig. 1. LPS profiles of *V. vulnificus* SerA isolates A1 (lane A), A8 (B), A9 (C), A14 (D), A15 (E) and 17 (F), SerO3 isolate 95-8-6 (G), SerO3/O4 isolate 95-8-161 (H), SerE isolate CECT 4604 (I) and BT3 isolate VV12 (J) visualized by immunostaining with anti-SerA serum diluted 1 : 1000. K, LPS profile of *V. vulnificus* SerE isolate CECT 4604 after immunostaining with its homologous antiserum diluted 1 : 1000.

larger than the high-*M_r* plasmid, around 69 kb, found in the SerE strain (Fig. 2). Most SerA isolates had a second large plasmid of around 50–65 kb (Fig. 2, Table 1). A third smaller plasmid of around 20–30 kb was present in two of the five groups established on the basis of the plasmid profiling (Table 1). The majority of isolates (groups 1 and 4) harboured three plasmids, whereas only one strain, Spanish isolate A8 (group 2), carried one plasmid, the common high-*M_r* one. Groups 3 and 4, comprising Spanish isolates recovered from the same outbreak in 2002, harboured a second plasmid of approximately 60–65 kb (Table 1). The non-motile strains isolated from wild eels displayed the group 1 plasmid profile (Fig. 2, Table 1). Thus, similar plasmid profiles were observed in isolates from different geographical origins and also in isolates recovered from either wild or cultured eels (Table 1). No plasmids were visualized in BT1 and BT3 control strains (Table 1).

The BT2-specific plasmidic sequence seq51 was searched for in SerA strains using PCR with the specific primer pair derived from the sequence. In the PCR assays, all SerA strains were positive, giving the amplification product specific for seq51, a band of 344 bp (data not shown).

Ribotyping

Since *V. vulnificus* biotypes and serovars are genotypically distinguishable by ribotyping (Aznar *et al.*, 1993; Biosca

Fig. 2. Plasmid profiles of *E. coli* 39R861 (lane A) and selected *V. vulnificus* SerA (lane B, A1-group 1; C, A8-group 2; D, A9-group 3; E, A14-group 4; F, 21A-group 5) and SerE (G, CECT4999) strains. Plasmids were extracted following basically the method of Zhou et al. (1990).

et al., 1997; Arias et al., 1997, 1998), this technique was applied to all isolates to investigate the genetic homology of SerA isolates. We selected ribotyping with *Hind*III because it displays the separation of the resulting fragments very well. Ribotyping of all SerA strains using *Hind*III yielded a common pattern consisting of eight bands ranging from 1.2 to 7.5 kb (Fig. 3). This ribopattern was different from those displayed by the SerE and type strains (Fig. 3). In these two different ribotypes, eight bands in the same range of molecular masses were also observed (Fig. 3).

Genetic relationships inferred by RAPD

The RAPD-PCR technique, based on primers M13 and T7, was applied to SerA isolates with the aim of detecting strain- or serovar-specific DNA profiles. RAPD-PCR with primer M13 and T7 of SerA isolates rendered reproducible patterns consisting of six to ten bands ranging from 270 to 1125 bp and from 341 to 1473 bp, respectively. Results obtained with both primers allowed the differentiation of the isolates at the intraspecific level and it was possible to distinguish individual strains within SerA. Fig. 4 shows the clusters defined at an 84 % similarity level by RAPD patterns obtained with primer M13. All SerA strains grouped together (cluster 1) and could be clearly separated from the type strain of the species and the reference SerE strain. At an 86 % similarity level, SerA strains were distributed in three subclusters, regardless of their geographical origin (Fig. 4).

Fig. 3. Ribotypes obtained with *Hind*III for representative *V. vulnificus* strains. Lanes: riboprinter molecular mass markers (values are given in kb on the left); A, SerA strain A1; B, SerE strain CECT 4604; C, BT1 strain ATCC 27562. Ribotyping was performed by automated ribotyping with the Riboprinter (Clermont et al., 2001).

A cluster analysis of RAPD profiles obtained with primer T7 revealed four groups of strains within SerA at an 84 % similarity level and two strains remained ungrouped (data not shown). As with primer M13, SerE strain showed a clearly different pattern from those of SerA. However, the type strain grouped with SerA strains (data not shown). Therefore, based on RAPD-PCR patterns, SerA strains can clearly be separated from SerE strain using primers M13 or T7, and also from type strain of BT1 if primer M13 is used.

Resistance to sera and virulence assays

All SerA strains could survive and grow in fresh eel serum, and most of them gave bacterial yields similar to those of the control SerE strain (Table 2). However, SerA strains did not resist the bactericidal action of fresh human serum, with a large reduction (≥ 2 log units) in the cell counts noticeable at the end of the experiments (Table 2).

As expected, all SerA isolates were virulent for eels after intraperitoneal injection, the majority of them with high LD₅₀s ranging from 3.0×10^1 to 6.2×10^2 c.f.u. per fish (Table 2). Moribund fish showed clinical signs of haemorrhagic septicaemia and *V. vulnificus* SerA was recovered from all of them in pure culture. The LD₅₀ of the most virulent SerA strains was even lower than that of the SerE used as control (Table 2). However, none of the tested SerA strains were virulent for mice or iron-overloaded

Fig. 4. Cluster analysis of the RAPD patterns obtained with universal primer M13 of *V. vulnificus* strains performed with Pearson coefficient and UPGMA. The banding pattern corresponding to each type is shown on the right. Subclusters are indicated with arrows.

mice. In fact, LD₅₀s were higher than 10⁷ or 10⁵ c.f.u. per mouse for untreated or Hb-treated animals in all cases (Table 2). No significant reduction in LD₅₀ was observed after iron treatment of mice, as occurred in SerE strain (Table 2).

DISCUSSION

SerA isolates were highly homogeneous as well as very sensitive to antibiotics, which is in accordance with the character of the SerA as an emergent pathogen. All strains

Table 2. Virulence for eels and mice, effect of iron treatment on susceptibility of mice to infection, and growth in eel and human sera of selected isolates

Strain*	Biotype/serovar	Survival in eel serum (GR)†	Virulence for eels (LD ₅₀)‡	Survival in human serum (GR)	LD ₅₀ (c.f.u. per mouse)\$	
					No treatment	Hb
A1, A3, A10, A12, A14, A15II, A17, A19, 19, 21A, 22, 26, 27	BT2/SerA	+(2)	$3.0 \times 10^1 - 6.2 \times 10^2$	–	$4.1 \pm 0.2 \times 10^7$	$>2.0 \times 10^5$
17	BT2/SerA	+(2)	6.0×10^3	–	3.3×10^7	$>2.0 \times 10^5$
A8	BT2/SerA	+(1)	8.0×10^4	–	8.0×10^7	$>2.0 \times 10^5$
A9	BT2/SerA	+(1)	7.0×10^5	–	NT	NT
CECT 4604	BT2/SerE	+(2)	5.0×10^1	+(2)	2.0×10^5	1.1×10^2

*CECT, Spanish Type Culture Collection; II non-motile strain.

†The final growth rate (GR) in serum was calculated for each strain as the log (final count/initial count) and was coded as 1 ($0 < \text{GR} \leq 1$) or 2 ($1 < \text{GR} \leq 2$).

‡LD₅₀ after intraperitoneal injection (c.f.u. per fish).

\$LD₅₀ after intraperitoneal injection for mice without and with iron (as haemoglobin, Hb) treatment before infection (Helms et al., 1984). NT, Not tested.

gave an identical API 20E profile to that of the type strain of the species and were correctly identified at species level. The profile differed from that of the SerE isolates in indole and ornithine decarboxylation tests, confirming the limited value of both assays as BT markers. The only exception was the motility characteristic, which was negative for two isolates from wild eels. The lack of motility has already been reported in other typically motile bacterial pathogens (Fouz *et al.*, 2006b; Gardel & Mekalanos, 1996), including *V. vulnificus* (Esteve *et al.*, 2007). The use of vaccination against *V. vulnificus* at farms (Esteve-Gassent *et al.*, 2004) could have favoured the emergence of these non-motile strains by selective pressure against flagellar antigens, similarly to what has occurred with other motile fish pathogens (Fouz *et al.*, 2006b). The new non-motile variant could have been transmitted to wild eels from cultured eels, set free in natural environments for restocking purposes.

Serological assays by slide-agglutination tests suggested that the SerA isolates constituted an antigenically homogeneous group, distinguishable from SerE. This finding was confirmed after LPS extraction and analysis by immunoblotting. Thus, all SerA strains exhibited identical LPS with a ladder-like structure that differed from that found in the control SerE strain and from those previously published for BT1, BT2-SerE and BT3 strains (Amaro *et al.*, 1992b; Biosca *et al.*, 1996; Bisharat *et al.*, 2007). In addition, no cross-reactions were detected between LPS from SerA and SerE strains, although some cross-reactions with anti-SerO3, -SerO3/O4 and -BT3 sera that affected the lipid A and core region of SerA-LPS were detected. These results could partially explain the slight cross-reactions obtained in agglutination tests among serovars A, O3 and O3/O4 or those previously found in dot blot assays between SerA and BT3 (Bisharat *et al.*, 2007). From these data, it has been concluded that the serological specificity of this new group would be based on the O-antigen of the LPS. This molecule could be a good candidate for the development of rapid methods to detect this emergent pathogen.

The presence of plasmids of high M_r in BT2 strains has previously been reported (Biosca *et al.*, 1997; Høi *et al.*, 1998; Lewin *et al.*, 2000). In fact, three BT2-specific plasmidic sequences have been found by comparison of BT1 and BT2 genomes by subtractive hybridization (Lee *et al.*, 2005). From these sequences, only one, seq51, was related to a putative open reading frame (*orf51*) (Lee *et al.*, 2005). The authors concluded that the SerE plasmid of about 68 kb, harbouring *orf51*, was related to eel virulence (Lee *et al.*, 2005; Amaro *et al.*, 2006). Results obtained in the present study are in accordance with the above hypothesis since all SerA strains (i) gave the expected product after PCR amplification with primers directed against *orf51* and (ii) harboured one apparently common large plasmid, which could be involved in the eel virulence of this emergent serovar. However, the plasmid profiles lacked epidemiological value since they did not enable isolates to be grouped on the basis of geographical origin

or source, as reported for SerE strains (Biosca *et al.*, 1997). The molecular size of the common SerA plasmid was slightly higher than that of the common SerE plasmid, which suggests that the two plasmids are not identical. Studies are under way concerning the homology between the high- M_r plasmid harboured by SerA and SerE strains.

All SerA strains, irrespective of their geographical origin, presented identical or nearly identical ribopatterns after *Hind*III digestion, which would suggest their genetic proximity. This pattern was different from that shown by control strains of BT1 and SerE [ribopattern that corresponded to the predominant one of this serovar (Arias *et al.*, 1997, 1998; Biosca *et al.*, 1997)]. The genetic proximity among SerA isolates was also observed by RAPD-PCR. In fact, SerA strains clustered together, separately from the SerE strain, on the basis of RAPD analysis. Of the two primers used, M13 corresponded optimally with the results obtained by ribotyping, as previously reported by other authors (Aznar *et al.*, 1993; Arias *et al.*, 1998). The homogeneity found in the ribopatterns and RAPD profiles with M13 among strains of SerA correlates with the serological results and suggests that all SerA isolates belong to the same clonal group and are different from SerE isolates. This heterogeneity had previously been found among eel isolates by other authors using different serovars and approaches, such as sequence typing, multilocus enzyme electrophoresis and RAPD (Gutacker *et al.*, 2003). Our data and those of these authors strongly suggest the existence of distinct genetic groups associated with disease in eels.

Finally, virulence assays revealed that all SerA isolates, including the non-motile ones, were highly virulent for eels but avirulent for mice, even after pretreatment with iron. These results correlated with those of resistance to the bactericidal activity of eel and human sera. Since resistance to the bactericidal action of serum is clearly related to the infective potential of this species (Amaro *et al.*, 1997), it is reasonable to speculate that SerA is unable to produce disease in humans. Furthermore, the tested SerA strains developed opaque colonies on conventional media, suggesting that the lack of virulence for mice or the inability to withstand human serum could not be explained by the absence of capsule as occurs in BT1 and BT2 SerE strains (Amaro *et al.*, 1994; Amaro & Biosca, 1996; Oliver, 1989; Strom & Paranjpye, 2000).

Conclusion

V. vulnificus SerA, a genetically distinct group within the species *V. vulnificus*, is highly virulent for eels but lacks potential to infect humans. This group could have evolved from another non-pathogenic one by horizontal acquisition of a fish-virulence plasmid. In order to elucidate this hypothesis, further studies on the genetic basis of the virulence potential of SerA are currently in progress.

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