Serological and molecular characteristics of *Vibrio vulnificus* biotype 3: evidence for high clonality

Naiel Bisharat,^{1†} Carmen Amaro,² Belén Fouz,² Amparo Llorens² and Daniel I. Cohen¹

Correspondence

Naiel Bisharat

bisharat_na@clalit.org.il

¹Department of Epidemiology and Preventive Medicine, School of Public Health, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv, Israel

²Departamento de Microbiología y Ecología, Campus de Burjassot, Universidad de Valencia, Valencia 46100, Spain

Vibrio vulnificus biotype 3 has been implicated as the causative pathogen of an ongoing disease outbreak that erupted in Israel in 1996. Recent work based on multi-locus sequence typing (MLST) showed that *V. vulnificus* biotype 3 is genetically homogeneous. The aim of this study was to investigate the existence of subpopulations within this homogeneous biotype by characterizing the surface antigens and analysing the sequence diversity of selected outer-membrane protein (OMP)-encoding genes. Rabbit antisera were prepared against biotype 1, 2 and 3 strains. The results of the slide-agglutination test, dot-blot assay (using fresh and boiled cells), and immunoblotting of lipopolysaccharides (LPS) and OMPs were evaluated. By slide-agglutination and dot-blot assays all biotype 3 strains agglutinated with the selected biotype 3 strain. This homogeneity was supported by immunoblot analysis of the LPS. Analysis of OMP patterns revealed that all three biotypes share a considerable number of common bands that are antigenically related. Cluster analysis of DNA sequence data from selected OMP-encoding genes showed that biotype 3 strains form a genetically distinct and homogeneous clone. The homogeneity of surface antigens and the lack of any sequence diversity among both housekeeping and OMP-encoding genes reaffirms the highly clonal nature of biotype 3 and suggests that it has only recently descended from the parent population of *V. vulnificus*.

Received 26 October 2006

Revised 26 November 2006

Accepted 6 December 2006

INTRODUCTION

Vibrio vulnificus is considered the major cause of seafood-related deaths, with high fatality rates, especially among immunocompromised hosts (Oliver, 1989). The bacterium also causes a fish disease designated vibriosis, causing considerable economical losses in European eel culture (Amaro *et al.*, 1992b). The species has been subdivided into three biotypes based on differences in biochemical, serological and molecular traits as well as host range (Bisharat *et al.*, 1999; Blake *et al.*, 1979; Dalsgaard *et al.*, 1996; Tison *et al.*, 1982). Worldwide, human disease is

almost entirely caused by biotype 1 strains, while biotype 2 has been mainly associated with disease in eels and rarely affecting humans, mainly by the most virulent of all biotype 2 serovars, serovar E (Dalsgaard *et al.*, 1996). Biotype 3 is geographically restricted to Israel, where it caused a serious outbreak of wound infections and bacteraemia among fish farmers and consumers of *Tilapia* fish cultivated in inland fish farms (Bisharat *et al.*, 1999). To date, biotype 3 has been the only variant isolated from human disease cases in Israel.

Several molecular techniques have been applied in an attempt to better characterize this emerging pathogen (biotype 3). By pulsed field gel electrophoresis (PFGE) the strains were non-typable (Bisharat *et al.*, 1999). PCR-RFLP (restriction fragment length polymorphism) of the cytotoxin-haemolysin gene showed that all biotype 3 strains were indistinguishable and distinct from biotypes 1 and 2 (Bisharat *et al.*, 1999). Similar results were obtained using multi-locus enzyme electrophoresis (Gutacker *et al.*, 2003). Recent work, based on multi-locus sequence typing (MLST) data revealed that biotype 3 is a recombinant clone that evolved by the hybridization between two populations (Bisharat *et al.*, 2005). The analysis also showed that all the

Part of this work was done at the Nuffield Department of Clinical Laboratory Sciences, University of Oxford, Oxford, UK.

[†]Present address: Department of Medicine, section D, Ha'Emek Medical Center, Afula 18101, Israel.

Abbreviations: MLST, multi-locus sequence typing; OMP, outer-membrane protein.

The GenBank/EMBL/DDBJ accession numbers for the *vuuA* sequences reported in this paper are DQ973529, DQ973530 and DQ980539; for the *wcvI* sequences, DQ980540 and DQ980541; for the *wza* sequences, DQ980542 and DQ980543; and for the *ompU* sequences, DQ980544, DQ980545 and DQ980546.

strains belonging to this clone were genetically uniform among all 10 housekeeping genes analysed.

The fundamental concept behind MLST is to analyse DNA sequence diversity within housekeeping genes encoding enzymes involved in intermediary metabolism, which are not subject to any unusual selective forces, and diversify slowly by the random accumulation of neutral mutations, and therefore better reflect the genetic population structure of the species (Maiden *et al.*, 1998). Alternatively, the competition between hosts and their pathogens offers clear opportunities for selection to play a prominent evolutionary role. Thus, many pathogens show antigenic surface variations as an adaptation mechanism to evade the immune system and successfully colonize host tissues (Parmley *et al.*, 1994; Renia *et al.*, 1997). Nucleotide sequence analysis and serological characterization of outer-membrane proteins (OMPs) may provide insights into the substructure of a homogeneous lineage (based on nucleotide sequence analysis of housekeeping genes) and may identify different subpopulations with an epidemiological interest.

The aim of the present work was to characterize the surface antigens, lipopolysaccharide (LPS) and OMPs of *V. vulnificus* biotype 3 and to analyse the sequence diversity of selected OMP-encoding genes.

METHODS

Bacterial isolates and culture media. The study collection consisted of 41 isolates of *V. vulnificus*, representing all three biotypes: biotype 1 ($n=18$), biotype 2 ($n=4$) and biotype 3 ($n=19$). The list of isolates and their sources is shown in Table 1. Strains were routinely cultured in Trypticasein Soy Agar (TSA) or Broth (TSB) (Pronadisa) supplemented with 0.5% (w/v) NaCl (TSA-1 or TSB-1) at 25 °C (biotype 2) or 37 °C (biotypes 1 and 3) for 24 h. For the analysis of the OMPs, cells were grown in Marine Sea Water Yeast Extract (MSWYE) (Oliver & Colwell, 1973) for 24 h. MSWYE is a medium that provides cells enough iron-restriction conditions to express iron-regulated OMPs (Esteve-Gassent & Amaro, 2004). Strains were stored in Marine Broth (Difco) plus 20% (v/v) glycerol at -80 °C.

Antisera. Anti-biotype 1, 2 and 3 rabbit sera were prepared by intravenous injection of New Zealand rabbits with formalin-killed cells from selected strains (ATCC 27562, biotype 1; CECT 4604, biotype 2, serovar E; and VV12, biotype 3) (see Tables 1 and 4) according to the procedure described by Sorensen & Larsen (1986). One week after the last injection, the rabbits were bled from the ear vein. All sera were stored in aliquots and frozen at -80 °C until used.

Serological testing

Slide-agglutination assay. The slide-agglutination assay was performed by mixing 20 µl of a cell suspension in sterile saline solution (SS, 0.9% NaCl, pH 7.4) (10^9 c.f.u. ml⁻¹) with 20 µl of serial two-fold dilutions of anti-biotype 1, 2 and 3 serum (Amaro *et al.*, 1992b). The agglutination assay was performed using fresh (thermolabile antigens) and boiled (at 100 °C for 2 h; thermostable antigens) cell suspensions. A distinct and immediate agglutination was defined as positive. Agglutination titre was recorded as the reciprocal of the highest serum dilution giving a positive reaction.

Dot-blot assay. Dot-blot analysis was performed using thermolabile and thermostable antigens as previously described (Cipriano *et al.*, 1985). Briefly, 1 µl volumes of antigens were dotted onto cellulose membranes (0.5 µm; Bio-Rad) and blocked for 1 h with 3% (w/v) skimmed milk in Tris-buffered saline (TBS) (50 mM Tris/HCl, pH 7.4). After washing twice with TBS supplemented with 0.05% Tween 20 (TBS-T), membranes were incubated for 1 h with serial dilutions from 1:1000 to 1:64 000 of anti-biotype 1, 2 and 3 sera in TBS containing 1% gelatin (TBS-1). After washing again, membranes were incubated with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Bio-Rad) diluted at 1:3000 in TBS-1 for 1 h. Specific antigen-antibody binding was detected by colour development with the substrate solution. *Escherichia coli* JB101 was used as a negative control. Serum titre was calculated for each antigen as the reciprocal of the lowest antibody dilution giving a similar response to that of the negative control.

Outer-membrane antigens. OMP and LPS samples were obtained from 24 h bacterial cultures in MSWYE medium as previously described (Amaro *et al.*, 1992a; Biosca *et al.*, 1993). Briefly, at mid-exponential phase the bacterial cells were harvested and disrupted by exposing to distilled water. The cell envelopes were collected by centrifugation at 30 000 g for 30 min and treated with a solution of Sarkosyl (sodium lauroyl sarcosinate) at 0.55% (w/v) for 10 min. After centrifugation at 18 000 g for 2 h (Biosca *et al.*, 1993), pellets were resuspended in distilled water. Protein concentration was measured by the Lowry method and adjusted to 1 mg ml⁻¹. Samples were mixed (1:1) with 2 × electrophoresis final sample buffer (FSB) (Laemmli, 1970), boiled for 5 min and stored at -20 °C. LPS extractions were prepared from whole-cell lysates according to the method of Hitchcock & Brown (1983) as modified by Amaro *et al.* (1992a). After proteinase K treatment (Boehringer Mannheim), samples were mixed (1:1) with 2 × FSB and boiled for 5 min.

SDS-PAGE and immunoblotting. LPS and OMP samples were analysed by SDS-PAGE (Laemmli, 1970) and immunoblotting according to previously described methods (Amaro *et al.*, 1992a; Biosca *et al.*, 1993; Esteve-Gassent & Amaro, 2004). OMPs were visualized by both Coomassie brilliant blue staining and immunostaining with anti-OmpU or anti-VuuA sera (Esteve-Gassent & Amaro, 2004), and LPS bands by immunostaining with anti-biotype serum (Amaro *et al.*, 1992a). Prior to immunostaining, protein and LPS bands were transferred from the polyacrylamide gels to nitrocellulose sheets (0.45 µm; Bio-Rad) as described by 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% methanol). The immunological staining of LPS and OMPs was performed using a 1:1000 dilution of rabbit anti-biotype 1, 2 and 3 sera, or a 1:4000 dilution of anti-OmpU/VuuA serum, respectively (Amaro *et al.*, 1992a; Esteve-Gassent & Amaro, 2004). Pre-stained molecular mass markers (Kaleidoscope Prestained Standards; Bio-Rad) were used as controls.

DNA extraction. The DNeasy kit (Qiagen) was used to extract DNA with the Gram-negative bacterial protocol as recommended by the manufacturer.

Genes chosen for analysis. The following OMP-encoding genes were chosen for further analysis: *wza*, encoding an outer-membrane lipoprotein involved in surface assembly of capsular antigen (Wright *et al.*, 2001); *ompU*, encoding a major OMP (Park *et al.*, 2006); *vuuA*, encoding the ferric vulnibactin outer-membrane receptor (Webster & Litwin, 2000); and *wcvI*, encoding a glycosyltransferase involved in capsule synthesis (Smith & Siebeling, 2003). Mutations in any of these selected genes have been related to some reduction of virulence of vibrios (Provenzano *et al.*, 2001; Smith & Siebeling, 2003; Webster & Litwin, 2000; Wright *et al.*, 2001).

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

Isolate*	Biotype†	ST‡	Country	Year	Source
ATCC 27562 ^T §	1	3	USA	–	Human
C7184	1	1	USA	–	Human
SPRC 10213	1	4	USA	–	Environment
960717-1/5C	1	10	Denmark	–	Diseased eel
WRI	1	12	USA	–	Environment
DAL 6-5000 (CDC 9345-95)	1	18	USA	1995	Human
LOS 7318 (CDC 9038-96)	1	20	USA	1996	Human
DAL 79040 (CDC 9070-96)	1	21	USA	1996	Human
M06	1	32	USA	–	Human
LAM264	1	32	USA	–	Human
99-625 DP-D8	1	38	USA	1999	Shellfish
99-537 DP-G7	1	39	USA	1998	Shellfish
I-29	1	47	Japan	–	Environment
5/I	1	58	Israel	1997	Environment
CCUG 38297	1	61	Sweden	1997	Human
ORL 8074 (CDC 9032-95)	1	64	USA	1995	Human
CH 1603 O:8	1	66	Germany	–	Environment
CCUG 47321	1	68	Sweden	2003	Human
CECT 4604 (E 86)§	2 (E)	6	Spain	1990	Diseased eel
CECT 4603 (E 58)	2 (E)	6	Spain	1990	Diseased eel
NCIMB 2136	2 (E)	6	Japan	1976	Diseased eel
CECT 5198	2 (non-E)	–	Spain	2000	Diseased eel
97	3	8	Israel	1997	Human
11028	3	8	Israel	1996	Human
VV12§	3	8	Israel	1996	Human
VV32	3	8	Israel	1996	Human
VV44	3	8	Israel	1998	Human
VV50	3	8	Israel	1999	Human
162	3	8	Israel	1997	Human
491771	3	8	Israel	1997	Human
407	3	8	Israel	1997	Human
8179	3	8	Israel	1997	Human
7356	3	8	Israel	1998	Human
658	3	8	Israel	1999	Human
58	3	8	Israel	1998	Human
2321	3	8	Israel	1997	Fish
ATCC BAA-86	3	8	Israel	1995	Human
VV 7-00	3	8	Israel	2000	Human
VV 3-01	3	8	Israel	2001	Human
VV 6-02	3	8	Israel	2002	Human
VV 4-03	3	8	Israel	2003	Human

*^T, type strain of the species; ATCC, American Type Culture Collection; CECT, Colección Española de Cultivos Tipo; CDC, Centers for Disease Control; CCUG, Culture Collection University of Goteborg (Sweden).

†E, serovar E; non-E, non-serovar E.

‡ST, sequence type.

§Strains used for rabbit immunization to obtain specific antisera.

The fragments sequenced from each of these genes are shown in Table 2. Primers were designed based on conserved regions from alignment of the nucleotide sequence of these genes from the two clinical strains of *V. vulnificus*, CMCP6 (GenBank accession nos AE016795 and AE016796) and YJ016 (GenBank accession nos

BA000037 and BA000038) (Chen *et al.*, 2003). The PCR and sequencing primers are shown in Table 3. For the *wza* and *wcvI* genes, the same primers were used for PCR amplification and sequencing (in different concentrations: for PCR amplification 100 pmol μl^{-1} and for sequencing 1 pmol μl^{-1}). For the *ompU* and

Table 2. Amplicon size and sequenced fragment of the chosen genes

Gene	Product	Size of amplified fragment (bp)	Size of sequenced fragment (bp)	Polymorphic sites (%)	d_N/d_S ratio
<i>wza</i>	Outer-membrane lipoprotein; surface assembly of capsular antigen	550	297	19 (6.4)	1.3
<i>ompU</i>	Outer-membrane protein U (porin)	1300	913	571 (56.4)	1.2
<i>vuuaA</i>	Ferric vulnibactin outer-membrane receptor (iron receptor)	2065	1628	247 (15.2)	1.5
<i>wcvI</i>	Glycosyltransferase	990	639	112 (17.5)	1.1

vuuaA genes, two sets of sequencing primers were used to sequence the desired size of the gene fragment. The PCR amplification, PCR product precipitation, sequencing reaction and sequencing precipitation methods were carried out as previously described for the MLST studies of *V. vulnificus* (Bisharat *et al.*, 2005). The sequences were assembled using the Staden suite of computer programs (Staden, 1996). The number of polymorphic nucleotide sites and the calculation of d_N/d_S ratio (non-synonymous/synonymous substitutions) were estimated using the program START (Jolley *et al.*, 2001). The phylogenetic relationships between the different strains were investigated using distance matrix methods, also known as clustering algorithmic methods [neighbour-joining (NJ) tree], as implemented in MEGA (molecular evolutionary genetic analysis) (<http://www.mega-software.net>) version 3.1 (Kumar *et al.*, 2004). The phylogenetic relationships between the different strains based on sequencing of OMP-encoding genes (current study) were compared with the phylogenetic relationships based on concatenated sequences from MLST data from a previous study (Bisharat *et al.*, 2005). For this analysis the nucleotide sequences of 10 MLST loci, making up the sequence type of each strain, were concatenated into a single (4326 bp) length of DNA representing each strain. The bootstrapping method was used to test the accuracy of the clustering. The values on the NJ tree are percentages of 500 computer-generated trees produced by randomly sampling the sequences and are shown at the nodes.

RESULTS

Serological characterization

All biotype 3 strains (six strains used for this part of the study), regardless of the antigen used, agglutinated with the

antiserum obtained against the selected biotype 3 strain (agglutination titres between 64 and 128) (Table 4). Conversely, they did not agglutinate (titres <2) with antisera against the selected biotype 1 and biotype 2-serovar E strains (Table 4). Likewise, biotype 1 (ATCC 27562) and biotype 2-serovar E (CECT 4604) strains only agglutinated with the homologous antisera (titres between 64 and 128) (Table 4). As expected, the biotype 2 strain of non-serovar E did not agglutinate with anti-CECT 4604 (serovar E) serum (Table 4).

The results of dot-blot assays using thermostable (boiled cells) and thermolabile (fresh cells) antigens from strains of the three biotypes are also shown in Table 4. Titres for each serum, evaluated with the homologous strain, were $\geq 64\,000$, regardless of the antigen used. The anti-biotype 3 serum gave the same titres with all biotype 3 strains, including the homologous strain (Table 4). Clear cross-reactions between biotypes were detected when thermolabile antigens were used (titres between 16 000 and 32 000) but not with thermostable antigens (titres = 2000) (Table 4).

OMP patterns from selected strains of the three biotypes are shown in Fig. 1. The OMP patterns of biotype 3 strains were almost identical and similar to those of the selected strains of biotype 1 and 2 (Fig. 1a). Part of the similarity was revealed by immunoblotting with specific antisera (Fig. 1b). Thus, a band that co-migrated as the protein OmpU and the other as

Table 3. Primers used for PCR amplification and sequencing

Locus	Use	Name and sequence of primer	
		Forward (5' to 3')	Reverse (5' to 3')
<i>wza</i>	Amplification and sequencing	wza-A1, ACCGTTCCCTTGAATACCCCA	wza-A2, GTTTCGCTGCACAAATCAAAGC
<i>ompU</i>	Amplification	ompU-A1, ATCGCACTTCTGTATCTGCTG	ompU-A2, AGAAGTCGTAACGTAGACCTAG
<i>vuuaA</i>	Amplification	VuuA-A1, ATGGCAGCTTTACGCCAG	VuuA-A2, ATGAAGCACCAATCATACGTGG
<i>wcvI</i>	Amplification and sequencing	wcvI-A1, GTTGTGATTGGTGAGACGATC	wcvI-A2, CGAGATTCAACAACCACACCA
<i>ompU</i>	Sequencing	ompU-S1, AAATCCAAGACGGCCTATACG	ompU-S2, AGAAGTCGTAACGTAGACCTAG
<i>ompU</i>	Sequencing	ompU-S3, GGCAGATAAAATCGCTGTTGC	ompU-S4, CCCATAGTGTAAAGACGCC
<i>vuuaA</i>	Sequencing	VuuA-S1, AGAGATGCCTGTTGTCGTTGT	VuuA-S2, CCAATTGAGCATTAGCGACCC
<i>vuuaA</i>	Sequencing	VuuA-S3, CAGCGAAACTATTACAAACCG	VuuA-S4, CGAATTGTAGCCTTTTGCCAC

Table 4. Titres of antisera raised against selected strains of *V. vulnificus* biotypes 1, 2 and 3 obtained by dot-blot and agglutination assays

The serovars are indicated in parentheses. ^T, type strain of the species. Dot-blot titre for each antiserum and antigen combination is expressed as the reciprocal of the highest dilution of serum giving a response above the negative control. Agglutination titre for each antiserum and antigen combination is expressed as the reciprocal of the highest dilution of serum giving a positive agglutination.

Strain and type of antigen used	Antiserum against					
	ATCC 27562 ^T (biotype 1)		CECT 4604 (biotype 2, serovar E)		VV12 (biotype 3)	
	Dot-blot titre	Agglutination titre	Dot-blot titre	Agglutination titre	Dot-blot titre	Agglutination titre
Biotype 1						
ATCC 27562 ^T						
Boiled cells	64 000	128	2000	<2	4000	<2
Fresh cells	> 64 000	128	32 000	<2	8000	<2
Biotype 2						
CECT 4603 (E)						
Boiled cells	4000	<2	64 000	64	4000	<2
Fresh cells	32 000	<2	> 64 000	128	4000	<2
CECT 4604 (E)						
Boiled cells	4000	<2	64 000	64	4000	<2
Fresh cells	32 000	<2	> 64 000	128	4000	<2
CECT 5198 (non-E)						
Boiled cells	2000	<2	2000	<2	4000	<2
Fresh cells	32 000	<2	32 000	<2	16 000	<2
Biotype 3						
VV12						
Boiled cells	2000	<2	2000	<2	64 000	128
Fresh cells	32 000	<2	16 000	<2	> 64 000	128
VV32						
Boiled cells	2000	<2	2000	<2	64 000	128
Fresh cells	16 000	<2	16 000	<2	> 64 000	128
VV44						
Boiled cells	2000	<2	2000	<2	64 000	64
Fresh cells	16 000	<2	16 000	<2	> 64 000	128
97						
Boiled cells	2000	<2	2000	<2	64 000	128
Fresh cells	16 000	<2	16 000	<2	> 64 000	128
162						
Boiled cells	2000	<2	2000	<2	64 000	64
Fresh cells	16 000	<2	16 000	<2	> 64 000	128
11028						
Boiled cells	2000	<2	2000	<2	64 000	128
Fresh cells	16 000	<2	16 000	<2	> 64 000	128

protein VuuA were identified in the OMP samples from all strains and biotypes (Fig. 1b). The band patterns of the three biotypes mainly differed in two major bands additional to OmpU, one of about 44 kDa, present in biotype 2 strain (lanes 1 and 2 in Fig. 1a) and the other of about 32 kDa, present in biotype 1 strain (lane 3 in Fig. 1a).

LPS of biotype 3 strains was visualized by immunostaining with anti-VV12 serum. All LPS samples from biotype 3 strains exhibited a ladder-like structure typical of smooth LPS (Fig. 2a). The pattern corresponding to O-antigen was clearly different from that exhibited by biotype 1 and 2 strains (Fig. 2b). Three fractions, of high, medium and low

mobility, were present in the biotype 3 pattern whereas only one, of medium and of very low mobility, was present in those of biotypes 1 and 2, respectively (Fig. 2b). Finally, LPS samples from biotype 3 strains were not stained with the antisera against the selected strains of biotypes 1 and 2 (Fig. 2b). Conversely, LPS samples from selected biotype 1 and 2 isolates were stained only with their homologous antisera but not with the anti-biotype 3 serum (Fig. 2b).

Estimation of sequence diversity

The proportion of the variable nucleotides present in the gene fragments (for all 19 strains used) ranged from 6.4 %

Fig. 1. (a) OMP patterns of strains of *V. vulnificus* biotypes 1, 2 and 3 grown in MSWYE and visualized by staining with Coomassie blue. Lanes: 1, CECT 5198 (biotype 2, non-serovar E); 2, CECT 4604 (biotype 2, serovar E); 3, ATCC 27562^T (biotype 1); 4, 97 (biotype 3); 5, 11028 (biotype 3); 6, VV12 (biotype 3); 7, VV32 (biotype 3); 8, molecular mass standards (Kaleidoscope Prestained Standards, Bio-Rad). BT, biotype. (b) Nitrocellulose filters from SDS-PAGE of OMP samples of strains ATCC 27562^T (biotype 1) (lanes 1, 4), CECT 4604 (biotype 2-serovar E) (lanes 2, 5) and VV12 (biotype 3) (lanes 3, 6) immunostained with antiserum against the purified putative vulnibactin receptor, VuuA (lanes 1, 2 3), and the main OMP, OmpU (lanes 4, 5, 6). The positions of two protein standards of 80 and 34.8 kDa are indicated. BT, biotype.

(*wza*) to 56.4 % (*ompU*) (Table 2). The proportion of nucleotide substitutions that changed the amino acid sequence (non-synonymous substitutions, d_N) and the proportion of silent changes (synonymous substitutions, d_S) were calculated for each fragment. Using these data, the d_N/d_S ratios were all above 1 (Table 2), suggesting that there

is a selection for a change in amino acid and consistent with the concept that OMP-encoding genes are not under a neutral selection process.

Nucleotide sequence analysis revealed that all biotype 3 strains had identical sequences at each of the four genes

Fig. 2. LPS patterns of strains of *V. vulnificus* biotypes 1, 2 and 3. (a) Nitrocellulose filters from SDS-PAGE of LPS samples immunostained with anti-biotype 3 serum diluted 1 : 1000. Lanes: 1, 97 (biotype 3); 2, 11028 (biotype 3); 3, VV12 (biotype 3); 4, VV32 (biotype 3); 5, 162 (biotype 3); 6, VV44 (biotype 3); 7, 58 (biotype 3); 8, ATCC BAA-86 (biotype 3). (b) Nitrocellulose filters from SDS-PAGE of LPS samples immunostained with antisera against biotype 1, 2 or 3 diluted 1 : 1000. Lanes: 1, ATCC 27562^T (biotype 1); 2, CECT 4604 (biotype 2-serovar E); 3, VV12 (biotype 3). BT, biotype.

examined. This result was in contrast to those of biotypes 1 and 2, for which almost every strain exhibited sequence diversity compared to the other strains of the homologous biotype. Cluster analysis of the individual genes produced two groups, yet the strains within the groups varied by gene (Fig. 3). It also showed that biotype 3 strains form a distinct and homogeneous clone, yet closely related to strains of biotypes 1 and 2, compatible with the OMP patterns obtained using SDS-PAGE (Fig. 1). For presentation purposes, only a subset of the strains was used for constructing the phylogenetic trees (11 biotype 1, 2 biotype 2 and 2 biotype 3 strains). For the *wcvI* gene only nine sequences are shown; the remaining sequences were

identical to the main group in the middle and for presentation purposes were taken out.

Correlation with analysis from MLST loci

Analysis of sequence data from these sites (OMP-encoding genes) showed little correlation with the analysis from MLST loci. Closely related strains (based on MLST analysis) did not exhibit similar genetic relatedness when OMP-encoding genes were analysed (Fig. 3). The only exception was biotype 3, which showed a distinct pattern of sequence homogeneity among both MLST loci and OMP-encoding genes. Nevertheless, the phylogenetic analysis using MLST

Fig. 3. Neighbour-joining tree of nucleotide sequences from four selected OMP-encoding genes.

data placed biotype 3 in an intermediate position between the two main populations of *V. vulnificus* (Fig. 4), whereas the analysis of OMP-encoding genes grouped biotype 3 with one of the two main clusters (Fig. 3).

DISCUSSION

The present study showed that *V. vulnificus* biotype 3 expresses a homogeneous LPS and exhibits highly conserved nucleotide sequences among OMP-encoding genes. Using polyclonal antibodies raised against whole cells, slide-agglutination and dot-blot assay showed that biotype 3 strains could be grouped together and be clearly distinguished from biotype 1 and 2 strains. Although agglutination titres were lower than dot-blot titres, both assays were highly discriminating, especially when boiled cells were used as antigens. Further serological characterization by immunostaining of LPS and OMP extracts showed that biotype 3 expresses a homogeneous LPS, whereas the OMP patterns of the three biotypes were related.

Analysis of DNA sequence diversity of selected OMP-encoding genes showed that biotype 3 strains form a genetically homogeneous clone, yet closely related to those of biotypes 1 and 2. These findings are compatible with the OMP patterns obtained (Fig. 1), suggesting that OMPs from the three biotypes are closely related. In fact, our goal of identifying subpopulations within the homogeneous biotype 3 was not achieved.

Elements suggestive of recent evolution from the parent population of *V. vulnificus* were associated with biotype 3: the lack of any genetic diversity among both housekeeping genes (Bisharat *et al.*, 2005) and OMP-encoding genes (present analysis), the distinct biochemical characteristics (Bisharat *et al.*, 1999) and the clear association with disease in humans. In fact, biotype 3 and biotype 2-serovar E share some interesting similarities, most remarkably the finding that they both express a homogeneous LPS (Biosca *et al.*, 1996 and present analysis), both are genetically homogeneous among MLST loci (Bisharat *et al.*, 2005, Fig. 4 and unpublished data), both exhibit a distinct biochemical pattern (Bisharat *et al.*, 1999; Tison *et al.*, 1982) and, finally, each has a very specific niche, biotype 2-serovar E primarily affecting eels and biotype 3 affecting only humans.

Maynard Smith *et al.* (Smith *et al.*, 1993) have suggested a model where clonal lineages emerge due to the acquisition of a strong selective advantage, which allows them to rise rapidly in frequency in the population, and as the strains diverge and specialize on their respective hosts, they become more reproductively isolated, eventually creating distinct lineages. In synthesizing these observations, we hypothesize that biotype 2-serovar E and biotype 3 have only recently descended from the parent population of *V. vulnificus*.

To date, the homogeneous biotype 3 has been isolated only in Israel. Data suggest that it has been circulating within Israeli fish farms at least since 1981 (Bisharat *et al.*, 1999).

Fig. 4. Neighbour-joining tree of concatenated sequences from 10 MLST loci (housekeeping genes). All biotype 3 strains resolved into the same sequence type. For clarity only one strain is shown.

Therefore, the exact cause for the disease outbreak in the 1990s has yet to be determined. Initially, it was suggested that changes in fish marketing procedures may have facilitated the disease outbreak (Bisharat & Raz, 1996). However, and despite the implementation of a new fish marketing policy, disease continued (N. Bisharat, unpublished data). A possible link between regional impact of global warming on the Eastern Mediterranean basin and disease outbreak has recently been suggested (Paz *et al.*, 2006).

In view of the trend of exporting stocks of cultured *Tilapia* fish (from Israel) the appearance of this virulent *V. vulnificus* biotype in other parts of the world should be considered. Interestingly, it has been speculated that the appearance of biotype 2 in European eel farms has probably occurred due to imports of carrier eels from Japan (Amaro *et al.*, 2001), where the biotype was originally described (Tison *et al.*, 1982).

The distinctiveness of the LPS of biotype 3 from the LPS of the other biotypes of *V. vulnificus* could be utilized for the development of rapid methods for the specific detection of this pathogen, as has been achieved for biotype 2 (Biosca *et al.*, 1997), and for the generation of LPS-based vaccines. The use of LPS-based vaccines in humans has not been successful so far, because of either the heterogeneity of the LPS molecule or the lack of sufficient immunogenicity, although recently some promising results were reported using immunogenic protein carrier covalently linked to *O*-deacylated lipopolysaccharide (LPS) derived from *Neisseria meningitidis* in animal models (Cox *et al.*, 2005). Thus far, the use of LPS-based vaccines against vibriosis in eels has not been promising and the authors suggested that LPS from *V. vulnificus* serovar E may not be immunogenic for eels (Collado *et al.*, 2000). Others, however, have reported more encouraging results against other vibrios (Fukuda & Kusuda, 1985; Salati & Kusuda, 1985). Studies are currently under way to determine whether the LPS molecule of *V. vulnificus* biotype 3 can elicit a significant immune response in humans (N. Bisharat, unpublished data).

In summary, the present work reaffirms the highly clonal nature of *V. vulnificus* biotype 3 and suggests that it has only recently evolved from the parent population. Future studies should focus on utilizing the present data for developing rapid detection methods, inquiring whether the LPS molecule is immunogenic in humans, and determining the feasibility and the effectiveness of LPS-based vaccines in humans.

ACKNOWLEDGEMENTS

This work was supported in part by the Wellcome Trust, grant no. 067147/Z/02/Z, and by the Spanish project AGL2005-04688 from the Spanish Ministry for Education and Science (SMES). B. Fouz thanks SMES for her research contract 'Ramón y Cajal'.

REFERENCES

- Amaro, C., Biosca, E. G., Fouz, B. & Garay, E. (1992a). Electrophoretic analysis of heterogeneous lipopolysaccharides from various strains of *Vibrio vulnificus* biotypes 1 and 2 by silver staining and immunoblotting. *Curr Microbiol* **25**, 99–104.
- Amaro, C., Biosca, E. G., Esteve, C., Fouz, B. & Toranzo, A. E. (1992b). Comparative study of phenotypic and virulence properties in *Vibrio vulnificus* biotype 1 and 2 obtained from a European eel farm experiencing mortalities. *Dis Aquat Organ* **13**, 29–35.
- Amaro, C., Fouz, B., Collado, R., Esteve-Gassent, D., Marcelo-Noales, E., Esteve, C., Barrera, R. & Alcaide, E. (2001). Design and development of a vaccine against eel vibriosis caused by *Vibrio vulnificus* serovar E (or biotype 2) applied to intensive culture facilities (Report). Valencia: Departamento de Microbiología y Ecología, Universidad de Valencia, Valencia, Spain.
- Biosca, E. G., Garay, E., Toranzo, A. E. & Amaro, C. (1993). Comparison of outer membrane protein profiles of *Vibrio vulnificus* biotypes 1 and 2. *FEMS Microbiol Lett* **107**, 217–222.
- Biosca, E. G., Oliver, J. D. & Amaro, C. (1996). Phenotypic characterization of *Vibrio vulnificus* biotype 2, a lipopolysaccharide-based homogeneous O serogroup within *Vibrio vulnificus*. *Appl Environ Microbiol* **62**, 918–927.
- Biosca, E. G., Marco-Noales, E., Amaro, C. & Alcaide, E. (1997). An enzyme-linked immunosorbent assay for detection of *Vibrio vulnificus* biotype 2: development and field studies. *Appl Environ Microbiol* **63**, 537–542.
- Bisharat, N. & Raz, R. (1996). *Vibrio* infection in Israel due to changes in fish marketing. *Lancet* **348**, 1585–1586.
- Bisharat, N., Agmon, V., Finkelstein, R., Raz, R., Ben-Dror, G., Lerner, L., Soboh, S., Colodner, R., Cameron, D. N. & other authors (1999). Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel. Israel *Vibrio* Study Group. *Lancet* **354**, 1421–1424.
- Bisharat, N., Cohen, D. I., Harding, R. M., Falush, D., Crook, D. W., Peto, T. & Maiden, M. C. (2005). Hybrid *Vibrio vulnificus*. *Emerg Infect Dis* **11**, 30–35.
- Blake, P. A., Merson, M. H., Weaver, R. E., Hollis, D. G. & Heublein, P. C. (1979). Disease caused by a marine *Vibrio*. Clinical characteristics and epidemiology. *N Engl J Med* **300**, 1–5.
- Chen, C. Y., Wu, K. M., Chang, Y. C., Chang, C. H., Tsai, H. C., Liao, T. L., Liu, Y. M., Chen, H. J., Shen, A. B. & other authors (2003). Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. *Genome Res* **13**, 2577–2587.
- Cipriano, R. C., Pyle, J. B., Starliper, C. E. & Pyle, S. W. (1985). Detection of *Vibrio anguillarum* antigen by the dot blot assay. *J Wildl Dis* **21**, 211–218.
- Collado, R., Fouz, B., Sanjuan, E. & Amaro, C. (2000). Effectiveness of different vaccine formulations against vibriosis caused by *Vibrio vulnificus* serovar E (biotype 2) in European eels *Anguilla anguilla*. *Dis Aquat Organ* **43**, 91–101.
- Cox, A., Zou, W., Gidney, M. A., Lacelle, S., Plested, J. S., Makepeace, K., Wright, J. C., Coull, P. A., Moxon, E. R. & Richards, J. C. (2005). Candidacy of LPS-based glycoconjugates to prevent invasive meningococcal disease: developmental chemistry and investigation of immunological responses following immunization of mice and rabbits. *Vaccine* **23**, 5045–5054.
- Dalsgaard, A., Frimodt-Møller, N., Bruun, B., Hoi, L. & Larsen, J. L. (1996). Clinical manifestations and molecular epidemiology of *Vibrio vulnificus* infections in Denmark. *Eur J Clin Microbiol Infect Dis* **15**, 227–232.

- Esteve-Gassent, M. D. & Amaro, C. (2004). Immunogenic antigens of the eel pathogen *Vibrio vulnificus* serovar E. *Fish Shellfish Immunol* 17, 277–291.
- Fukuda, Y. & Kusuda, R. (1985). Vaccination of Yellowtail against pseudotuberculosis. *Fish Pathol* 20, 421–425.
- Gutacker, M., Conza, N., Benagli, C., Pedroli, A., Bernasconi, M. V., Permin, L., Aznar, R. & Piffaretti, J. C. (2003). Population genetics of *Vibrio vulnificus*: identification of two divisions and a distinct eel-pathogenic clone. *Appl Environ Microbiol* 69, 3203–3212.
- Hitchcock, P. J. & Brown, T. M. (1983). Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol* 154, 269–277.
- Jolley, K. A., Feil, E. J., Chan, M. S. & Maiden, M. C. (2001). Sequence type analysis and recombinational tests (START). *Bioinformatics* 17, 1230–1231.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 5, 150–163.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Unwin, R., Zhang, Q., Zhou, J., Zurth, K. & other authors (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* 95, 3140–3145.
- Oliver, J. D. (1989). *Foodborne Bacterial Pathogens*, pp. 569–600. Edited by M. P. Doyle. New York: Marcel Dekker.
- Oliver, J. D. & Colwell, R. R. (1973). Extractable lipids of gram-negative marine bacteria: phospholipid composition. *J Bacteriol* 114, 897–908.
- Park, D. K., Lee, K. E., Baek, C. H., Kim, I. H., Kwon, J. H., Lee, W. K., Lee, K. H., Kim, B. S., Choi, S. H. & Kim, K. S. (2006). Cyclo(Phe-Pro) modulates the expression of ompU in *Vibrio* spp. *J Bacteriol* 188, 2214–2221.
- Parmley, S. F., Gross, U., Sucharczuk, A., Windeck, T., Sgarlato, G. D. & Remington, J. S. (1994). Two alleles of the gene encoding surface antigen P22 in 25 strains of *Toxoplasma gondii*. *J Parasitol* 80, 293–301.
- Paz, S., Bisharat, N., Paz, E., Kidar, O. & Cohen, D. (2006). Climate change and the emergence of *Vibrio vulnificus* disease in Israel. *Environ Res* (in press) <http://www.sciencedirect.com/science/journal/00139351>.
- Provenzano, D., Lauriano, C. M. & Klose, K. E. (2001). Characterization of the role of the ToxR-modulated outer membrane porins OmpU and OmpT in *Vibrio cholerae* virulence. *J Bacteriol* 183, 3652–3662.
- Renia, L., Ling, I. T., Marussig, M., Miltgen, F., Holder, A. A. & Mazier, D. (1997). Immunization with a recombinant C-terminal fragment of *Plasmodium yoelii* merozoite surface protein 1 protects mice against homologous but not heterologous *P. yoelii* sporozoite challenge. *Infect Immun* 65, 4419–4423.
- Salati, F. & Kusuda, R. (1985). Vaccine preparations used for immunization of eel *Anguilla japonica* against *Edwardsiella tarda* infection. *Bull Jpn Soc Sci Fish* 51, 1233–1237.
- Smith, A. B. & Siebeling, R. J. (2003). Identification of genetic loci required for capsular expression in *Vibrio vulnificus*. *Infect Immun* 71, 1091–1097.
- Smith, J. M., Smith, N. H., O'Rourke, M. & Spratt, B. G. (1993). How clonal are bacteria? *Proc Natl Acad Sci U S A* 90, 4384–4388.
- Sorensen, U. B. & Larsen, J. L. (1986). Serotyping of *Vibrio anguillarum*. *Appl Environ Microbiol* 51, 593–597.
- Staden, R. (1996). The Staden sequence analysis package. *Mol Biotechnol* 5, 233–241.
- Tison, D. L., Nishibuchi, M., Greenwood, J. D. & Seidler, R. J. (1982). *Vibrio vulnificus* biogroup 2: new biogroup pathogenic for eels. *Appl Environ Microbiol* 44, 640–646.
- Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76, 4350–4354.
- Webster, A. C. & Litwin, C. M. (2000). Cloning and characterization of *vuuA*, a gene encoding the *Vibrio vulnificus* ferric vulnibactin receptor. *Infect Immun* 68, 526–534.
- Wright, A. C., Powell, J. L., Kaper, J. B. & Morris, J. G., Jr (2001). Identification of a group 1-like capsular polysaccharide operon for *Vibrio vulnificus*. *Infect Immun* 69, 6893–6901.

Edited by: P. H. Everest