VNIVERSITAT DE VALÈNCIA

DEPARTAMENTO DE MICROBIOLOGIA Y ECOLOGIA

Epidemiology and phylogeny of *Vibrio vulnificus* biotype 2

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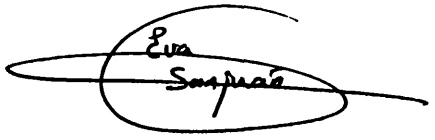
A thesis submitted to the University of Valencia
in fulfillment of the requirements for the degree
of Doctor of Philosophy in Biology



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CERTIFICA:

Que la presente Tesis Doctoral titulada "Epidemiology and phylogeny of *Vibrio vulnificus* biotype 2", que presenta **Eva Sanjuán Caro** para optar al grado de Doctor en Biología ha sido realizada en la Unidad de Microbiología de la Facultad de Ciencias Biológicas.

Y para que así conste a todos los efectos oportunos expido el presente certificado en Valencia, a 9 de Noviembre de 2007.

Fdo. Dra. M. Rosa Miracle Solé

A mis padres y mi hermana

A mis amigos

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LIST OF MOST USED ABBREVIATION

Bacterial type collections

ATCC American Type Culture Collection
CECT 'Colección Española de Cultivos Tipo'. Spanish Type Culture Collection
NCIMB National Collections of Industrial, Marine and Food Bacteria
CCUG Culture Collection, University of Göteborg
CIP Collection Institute Pasteur
^{T}. Determine the type culture of the bacterial specie

Others

PCR Polymerase Chain Reaction
DNA Deoxyribonucleic Acid
RNA. Ribonucleic Acid
Bt1 Biotype 1
Bt2 Biotype 2
Bt2-SerE Biotype 2, Serovar E strains
BT2-nonSerE Biotype 2, Non serovar E strains
BT3 Biotype 3
ND No determined
CFU. Colony Forming Units

Abstract

Vibrio vulnificus is a heterogeneous gram-negative bacterium from brackish and saline water. The species comprise avirulent and human/fish virulent strains from environment or clinical sources. Based on some phenotypic differences the species has been subdivided into three biotypes; biotype 1 includes environmental (water, selfish...) and human strains isolated worldwide, biotype 2 clinical strains from fish and a few human strains (serovar E isolates) all of them isolated worldwide, and biotype 3 clinical strains from human outbreaks geographically restricted to Israel. However, this classification does not reflect the real situation of the species as the characters used for the biotyping are not longer useful for the correct characterization of new isolates. The goal of this work was to analyze the real structure of the species and the relationships between the isolates of the different biotypes.

To this end, we firstly increased our *V. vulnificus* collection with strains of the three biotypes isolated from different sources and geographical regions. We focused our work on the biotype 2 of the species because its high incidence in eel culture in Spain. This biotype was considered an obligate eel pathogen although previous works of our group demonstrated that this pathogen was able to survive for long periods of time in the water. Our first objective was developing a new isolation protocol that allows the specific isolation of the biotype 2 strains from the environment. Using eel serum in a saline buffer we were able to isolate biotype 2 strains from different water samples as well as from healthy eels. In parallel to this studies, we performed a genomic comparison (SSH technique) between biotype 1 and 2 strains in order to found some genetic loci related to host specificity. These analyses were performed in a collaborative project between our group and the laboratory of the Dr. Lien-I Hor (Tainan, Taiwan). Few differences were found, emphasizing the high similarity between biotypes 1 and 2 of *V. vulnificus*. Although this, we found three sequences specific for biotype 2 isolates and three more for serovar E isolates. The first ones are located in a plasmid that later studies have demonstrated that is essential for host specificity and fish virulence. Using some of these sequences we designed a Multiplex PCR assay that permits the rapid identification of the species, the biotype 2 and the zoonotic serovar.

Once we increased the genetic diversity of our collection we carry out different analysis that included phenotypic, molecular (ribotyping) and genetic (selected genetic typing and MLST of housekeeping and virulence genes) tests for typing bacterial isolates. The results clearly demonstrate that the species is highly

heterogeneous. Phenotipically we were not able to find a specific profile that could be associated with biotype, serovar, or origin of the isolate. The DNA polymorphisms studied prone to divide the *V. vulnificus* into two groups, one of them mainly associated with human septicaemic isolates and the other with environmental ones. Similar divisions were observed with the other techniques. Based on the results of these genetic studies we could conclude that the biotype 1 is the most heterogeneous, whereas the biotype 3 is quite homogeneous forming a separated clade that could have evolved recently. Regarding the biotype 2, the phylogenies obtained revealed that those isolates could have emerged from different environmental isolates and that the fish virulence would have been acquired with the virulence plasmid.

In conclusion, our study does not support the actual subdivision of the species into biotypes. Our data show that there are two distinct *V. vulnificus* populations that correlated very well with all the genetic typing methods applied. These two divisions could be suitable for a new classification scheme of the species.

Sinopsis

Vibrio vulnificus es una bacteria gram negativa, heterogénea de ambientes marinos y salobres. La especie esta constituida por cepas avirulentas y virulentas para el hombre o peces que se aíslan del ambiente o de muestras clínicas. Basado en varias diferencias fenotípicas, la especie se subdivide en tres biotipos; el biotipo 1 esta formado por aislados ambientales (agua, marisco...) y muestras clínicas humanas asiladas alrededor del mundo, el biotipo 2 son muestras clínicas aisladas de peces y unas pocas humanas (aislados de la serovariedad E) con una distribución mundial. Y el biotipo 3 son muestras clínicas de brotes infecciosos en humanos limitados geográficamente a Israel. No obstante, esta clasificación no refleja la verdadera situación de la especie dado que los caracteres usados para el biotipado no son validos cuando se intenta caracterizar los nuevos aislados. El objetivo de este trabajo fue analizar la verdadera estructura de la especie así como las relaciones entre los aislados de los diferentes biotipos.

Para este propósito, primero incrementamos nuestra colección de cepas de *V. vulnificus* de los tres biotipos aislados de diferentes muestras y orígenes geográficos. Hemos centrado el trabajo en el biotipo 2 de la especie puesto que tiene gran incidencia en al anguilicultura en España. Este biotipo era considerado un patógeno obligado debido a la ausencia de aislados ambiental, pero trabajos publicados por nuestro grupo demostraron que este patógeno puede sobrevivir en el agua durante largos periodos de tiempo. Nuestro primer objetivo fue, por lo tanto, el desarrollo de un protocolo especifico de aislamiento de muestras ambientales del biotipo 2. Usando suero de anguila en un tampón salino fuimos capaces de aislar el biotipo 2 de diferentes muestras de agua y de anguilas sanas. En paralelo con este estudio, realizamos una comparación genómica (Técnica SSH) entre cepas del biotipo 1 y del 2 intentando encontrar algún loci genético que se relacionase con la especificidad de hospedador. Estos análisis se realizaron en un proyecto de colaboración entre nuestro grupo y el laboratorio de la Dra. Lien-I Hor (Tainan, Taiwán). Se encontraron pocas diferencias, lo que remarca el gran parecido entre ambos biotipos. Pero, pese a esto, encontramos 3 secuencias que eran especificas del biotipo 2 y otras 3 que estaban presentes solo en las cepas de la seriovariedad E. Las primeras están localizadas en un plásmido que posteriormente se ha demostrado que es esencial para la virulencia en peces. Usando algunas de estas secuencias diseñamos una PCR múltiple que permite la caracterización rápida y conjunta de la especie, del biotipo y de la serovariedad zoonótica.

Una vez incrementa la diversidad génica de nuestra colección llevamos a cabo diferentes análisis que incluían test fenotípicos, moleculares (ribotipado) y genéticos (análisis de la secuencia de genes de mantenimiento celular y de virulencia junto con el tipado génico de varios loci) de tipado bacteriano. Estos resultados demostraron claramente la heterogeneidad de la especie. Fenotípicamente no pudimos encontrar un perfil específico que pudiera asociarse a biotipo, serovariedad u origen de la cepa. El polimorfismo en varios genes estudiados tiende a dividir las muestras de *V. vulnificus* en dos grupos, uno de ellos asociados con aislados de septicemia humana y el otro más ambiental. Divisiones similares a esta se observaron con las otras técnicas usadas (ribotipado y MLSA). Basado en todos estos resultados pudimos concluir que el biotipo 1 es el más heterogéneo, mientras que el biotipo 3 es muy homogéneo formando un clado que ha evolucionado recientemente. En cuanto al biotipo 2, las filogenias muestran que estos aislados han emergido de diferentes muestras ambientales y que la virulencia para peces podría adquirirse al incorporar el plásmido de virulencia.

En conclusión, nuestro estudio no apoya la actual división de la especie en biotipos. Nuestros datos indican que hay dos poblaciones distintas dentro de V. vulnificus que se correlacionan bien con todas las técnicas de tipado genético empleadas. Estas dos divisiones podrían ser la base un nuevo esquema de clasificación de la especie.

CONTENTS

1

GENERAL INTRODUCTION

A GLOBAL PERSPECTIVE OF THE GENUS VIBRIO

According to Bergey's Manual of Systemic Bacteriology (2005), Vibrios (genus *Vibrio*) belongs to the Gammaproteobacteria and, are bacteria gram-negative straight or curved rod and motile by at least one polar flagellum. They are chemoorganotrophs, and facultative anaerobes, having both respiratory and fermentative metabolism. Furthermore, they are halophilic and with the exception of *V. cholerae* and *V. mimicus*, all *Vibrio spp.* require sodium ions (Na^+) for growth (Farmer, 2005). Nowadays, the genus contains more than 60 species (see table 1). Its taxonomy is being continuously updated as new species are described. In the last edition of the Bergey's, the family *Vibrionaceae* is divided into three genera: *Vibrio* (the type genus), *Photobacterium* and *Salinivibrio* (Farmer, 2005). Recently, Thompson *et al.*, based on the concatenated 16S rRNA, *recA* (recombinase A), and *rpoA* (RNA polymerase α subunit) gene sequence together with some phenotypic tests, proposed that the genus should be split into four new families (Thompson *et al.*, 2004):

- *Enterovibrionacea* that comprises two genera; *Enterovibrio* and *Grimontia.*
- *Photobacteriaceae* formed by the genus *Photobacterium.*
- *Salinivibrionaceae* comprising the genus *Salinivibrio.*
- *Vibrionaceae* that include the genus *Vibrio.*

The authors indicated that this division could facilitate further studies and suggested that the sequence of the 16s rRNA could be used for the allocation of a strain into a family while the species allocation could be obtained by other genetic data such as the AFLP (Amplified Fragment Length Polymorphism), rep-PCR (repetitive extragenic palindromic PCR) or the new MLSA (Multilocus sequence analysis) scheme proposed (Thompson *et al.*, 2005).

Table 1. Vibrios species described until December 2006

Species name	Authors and year	Pathogenic [a]	Salinity range (% NaCl)	Temperature range (ºC)
Vibrio aerogenes	Shieh *et al.* 2000	-	1-7	20-35
Vibrio aestuarianus	Tison and Seidler 1983	-	0.3-8	4-35
Vibrio agarivorans	Macián *et al.* 2001	-	0-6	20-37
Vibrio alginolyticus	(Miyamoto *et al.* 1961) Sakazaki 1968	H, F,AI, C	0.4->12	20-40
Vibrio anguillarum	Bergeman 1909	F	0.2-6	4-35
Vibrio brasiliensis	Thompson *et al.* 2003	F*,AI*	1-6*	20-40
Vibrio calviensis	Denner *et al.* 2002		1-12*	4-35
Vibrio campbellii	(Baumann *et al.* 1971) Baumann *et al.* 1980	C*	0.5-8	20-35
Vibrio chagasii	Thompson *et al.* 2003	-	1-8	4-30
Vibrio cholerae	Pacini 1854	H,F	0-6	20-40
Vibrio cincinnatiensis	Brayton *et al.* 1986	H	0.1-10	20-35
Vibrio coralliilyticus	Ben-Haim *et al.* 2003	C,F*,AI	1-8	Optimal 30 35

Species name	Authors and year	Pathogenic [a]	Salinity range (% NaCl)	Temperature range (ºC)
Vibrio crassostreae	Faury *et al.* 2004	AI	1-6	20-30
Vibrio cyclitrophicus	Hedlund and Staley 2001	-	2-10	4-37
Vibrio diabolicus	Raguénès *et al.* 1997	-	1-7	20-45
Vibrio diazotrophicus	Guerinot *et al.* 1982	-	0.1-10	4-35
Vibrio ezurae	Sawabe *et al.* 2004a	F*,AI*	1-6	4-40
Vibrio fischeri	(Beijerinck 1889) Lehmann and Neumann 1896	F	1-6	20-30
Vibrio fluvialis	Lee *et al.* 1981	H	0-10	20-40
Vibrio fortis	Thompson *et al.* 2003c	C*,F*,AI*	1-8	4-35
Vibrio furnissii	Brenner *et al.* 1984	H	0->12	20-40
Vibrio gallicus	Sawabe *et al.* 2004b	-	2-5	15-30
Vibrio gazogenes	(Harwood *et al.* 1980) Baumann *et al.* 1980	-	0.4->12	20-40
Vibrio gigantis	Roux *et al.* 2005	-	0-5	4-30

Species name	Authors and year	Pathogenic [a]	Salinity range (% NaCl)	Temperature range (ºC)
Vibrio halioticoli	Sawabe *et al.* 1998	-	3	15-30
Vibrio harveyi	(Johnson and Shunk 1936) Baumann *et al.* 1980	H, F,AI	1-8	20-35
Vibrio hepatarius	Thompson *et al.* 2003c	-	0-8	4-35
Vibrio hispanicus	Gomez-Gil *et al.* 2004	-	0-10	4-40
Vibrio ichthyoenteri	Ishimura *et al.* 1996	F	1-6	15-30
Vibrio kanaloae	Thompson *et al.* 2003c	F*,AI	1-8	4-30
Vibrio lentus	Macián *et al.* 2001a	-	1-6	4-30
Vibrio logei	(Harwood *et al.* 1980) Baumann *et al.* 1980	F	0.5-5	4-25
Vibrio mediterranei	Pujalte and Garay 1986	C	0.3-8	18-35
Vibrio metschnikovii	Gamaleia 1888	H	0.1-10	20-40
Vibrio mimicus	Davis *et al.* 1982	H,F	0-6	20-40
Vibrio mytili	Pujalte *et al.* 1993	-	0.4-10	10-37
Vibrio natriegens	(Payne *et al.* 1961) Baumann *et al.* 1981	-	0.5->12	20-40

Species name	Authors and year	Pathogenic [a]	Salinity range (% NaCl)	Temperature range (ºC)
Vibrio navarrensis	Urdaci *et al.* 1991	-	1-10	10-42
Vibrio neonatus	Sawabe *et al.* 2004	-	1-6	4-40
Vibrio neptunius	Thompson *et al.* 2003b	F*,AI*	0-8	4-40
Vibrio nereis	(Harwood *et al.* 1980) Baumann *et al.* 1981	-	1-10	4-40
Vibrio nigripulchritudo	(Baumann *et al.* 1971) Baumann *et al.* 1981	AI	1-6	20-30
Vibrio ordalii	Schiewe *et al.* 1982	F	0.5-6	4-20
Vibrio orientalis	Yang *et al.* 1983	-	0.5-8	4-35
Vibrio pacinii	Gomez-Gil *et al.* 2003a	-	1.5-8	4-35
Vibrio parahaemolyticus	(Fujino *et al.* 1951) Sakazaki *et al.* 1963	H,F	0.4-10	20-40
Vibrio pectenicida	Lambert *et al.* 1998	AI	1-3.5	20-30
Vibrio pelagius	(Baumann *et al.* 1971) Baumann *et al.* 1981	AI,F	0.5-8	4-35
Vibrio penaeicida	Ishimaru *et al.* 1995	AI	1-3.5	
Vibrio pomeroyi	Thompson *et al.* 2003d	-	1-8	4-30

Species name	Authors and year	Pathogenic [a]	Salinity range (% NaCl)	Temperature range (ºC)
Vibrio ponticus	Macián *et al.* 2004	-	1-8	4-35
Vibrio proteolyticus	(Merkel *et al.* 1964) Baumann *et al.* 1981	AI	0.2->12	20-40
Vibrio rotiferianus	Gomez-Gil *et al.* 2003[b]	C*,F*,AI*	1.5-6	28-40
Vibrio ruber	Shieh *et al.* 2003	-	1-10	20-40
Vibrio rumoiensis	Yumoto *et al.* 1999	-	3-6	2-34
Vibrio salmonicida	Egidius *et al.* 1986	F	0.5-5	4-22
Vibrio scophthalmi	Cerdà-Cuéllar *et al.* 1997	-	1-6	22-35
Vibrio splendidus	(Beijerinck 1900) Baumann *et al.* 1981	F,AI	1-8	4-35
Vibrio superstes	Hayashi *et al.* 2003	--	3	1.5-30
Vibrio tapetis	Borrego *et al.* 1996	AI	1-6	4-22
Vibrio tasmaniensis	Thompson *et al.* 2003[e]	-	1-6	4-35
Vibrio tubiashii	Hada *et al.* 1984	F,AI	0.3-8	10-35
Vibrio vulnificus	(Reichelt *et al.* 1979) Farmer 1980	H,F,AI	0.1-6	10-42

Species name	Authors and year	Pathogenic [a]	Salinity range (% NaCl)	Temperature range (ºC)
Vibrio wodanis	Lunder *et al.* 2000	F	1-4	4-25
Vibrio xuii	Thompson *et al.* 2003b	-	1-8	20-40

[a]: H, Human. F, Fish. AI, Aquatic invertebrate. C, Coral. Infection reported at least in one publication.(Thompson, 2006)

*: The pathogenic character has been suggested in the literature (Austin *et al.*, 2005; Thompson *et al.*, 2004)

It is well known that vibrio species are ubiquitous and widely distributed in aquatic environments worldwide. Their presence in these habitats is usually dependent on Na^+ and nutrient content as well as temperature. Vibrio species constitute a major group of culturable bacteria in marine and estuarine environments, mainly coastal waters.

According to some cultured-based studies, vibrios comprise more than 10% of the total of cultured bacteria. However, their number in the total bacterioplanckton is lower than 1% (Thompson, 2006). In general, they are frequently detectable in summer, while during winter months their isolation is more difficult. It is recognised that most vibrios enter in a viable but nonculturable (VBNC) state when exposed to low temperatures or otherwise adverse conditions. In this state, the bacterial cells are able to maintain metabolic activity (viability) although they are unable to grow on/in routine media (non-culturability) (Oliver, 1995; Oliver *et al.*, 1991; Thompson, 2006).

Bacteria of the genus *Vibrio* are able to synthesise different enzymes, including proteases, lipases, and chitinases that help in the degradation of complex polymers as part of the macronutrient cycles. Moreover, because the vibrios appear to be selectively grazing by flagellates, it had been suggested that these bacteria may

have enhanced significance in the cycling of organic matter. Same function has been also proposed for the viral mortality that could control the vibrios population. In addition, vibrio cells also participate in the fixation of nitrogen and the phosphorus cycle. Alkaline phosphatase, which is present in several vibrios, has been suggested to be central to supplying phosphate pools when phosphorous is limiting (Thompson, 2006).

Several studies showed that vibrios are highly abundant in or on marine organisms, They have been isolated from the surface of coral, fish, molluscs, seagrass, sponges, shrimp and zooplankton and also from the intestinal tract of some of these organisms (Thompson *et al.*, 2004). Different relationships could be established between a *V. spp.* and eukaryotic organisms. Symbiotic associations have also been described, such as the one between *V. fischeri* and squid species (Nyholm and McFall-Ngai, 2004; Ruby, 1996). The occurrence of the vibrios in the gut of some animals suggests a possible commensal role as they are able to degrade some polymeric compounds in a similar manner as the enteric bacteria (Jean, 1993). But, perhaps the most studied is the pathogenic relationship of several vibrios with a wide range of hosts (table 1).

Human diseases

To date, twelve vibrio species have been recognized to cause human illnesses (Dalsgaard, 1998; Thompson *et al.*, 2004). The list includes *Photobacterium damsela* (formerly *V. damsela*) and *V. hollisae* (reclassified as *Grimontia hollisa* (Thompson *et al.*, 2003a)). Of these human pathogens, *V. cholerae*, *V. paraheamolyticus* and *V. vulnificus* are the most important ones, both in number and severity of cases. Vibrio infections are generally divided into three recognized clinical syndromes (table 2):

i) Gastroenteritis, which is associated with the ingestion of contaminated raw or undercooked seafood and shellfish. The major

symptoms are diarrhoea, vomiting and abdominal cramps. Normally, neither antibiotic treatment nor hospitalization are required.

ii) Wound infection. Vibrio could infect a pre-existing wound (cut, mosquito bite, laceration...) or one incurred while fishing doing other coastal recreational activities or handling finfish or seafood. Some infections are self-limiting whereas others require medical assistance.

iii) Primary septicaemia, a systemic illness caused by bacteria entering the bloodstream. The symptoms are fever, hypotension, prostration, chills and occasionally abdominal pain, nausea, vomiting and diarrhoea. *V. vulnificus* is the most frequently isolated bacteria from patients with vibrio septicaemia (Tantillo *et al.*, 2004).

Many researches believe that the epidemiological data on vibrio infections might not represent the real situation. The mild or subclinical presentation of some of the infections, the difficulties in performing correct diagnoses (due to lack of information, wrong identification using rapid identification systems...) and the lack of an efficient surveillance system for vibrio infection could be the causes of this underestimation (Osaka *et al.*, 2004; Tantillo *et al.*, 2004).

Table 2. Human syndromes caused by vibrio infection

	INTESTINAL INFECTION	EXTRAINTESTINAL INFECTION		
Vibrio species	Gastroenteritis	Primary septice-mia	Wound infection	Ear/eye infect.
V. cholerae O1/O139	++		+	
V. cholerae non-O1	++	+	++	
V. parahaemolyticus	++	+	+	+
V. vulnificus	++	++	++	+
V. mimicus	++	+	++	
V. (Grimontia) hollisae	++	+	+	
V. fluvialis	++	+	+	+
V. alginolyticus	+		++	++
V. (Photobacterium) damselae		+	++	
V. metschnikovii	+	+	+	
V. cincinnatiensis	+	+		
V. harveyi			+	++
V. furnissii	+			

++: Infection reported, most common association. +: infection reported

Non Human diseases

Vibrios are abundant in the aquatic environment, causing diseases both in wild and cultured fish and shellfish including coral (Table 3). The diseases are generally known as "vibriosis".

Fish vibriosis is one of the most extended and prevalent fish diseases. Distribution is worldwide, mainly affecting marine and brackish water species although a few records of vibrio infections in freshwater species have also been reported (Austin and Austin, 1999). When vibriosis is produced in fish culture facilities, the disease can cause significant mortality (≥50%) and high morbidity (up to 100%) as bacteria spreads rapidly when fish are confined in heavily stocked commercial systems. Fish vibriosis is generally a septicemic disease with symptoms similar to other bacterial septicaemia. It usually starts with lethargy and loss of appetite. The skin may become decoloured and lesions appear that normally ulcerate and even necrotise. Erythemas are common around the fins and mouth. When the disease become systemic, it causes exopthalmia, the gut and rectum may be bloody and filled with fluid. In some cases there are also internal haemorrhages. The most important agent of vibriosis in fish is *V. anguillarum* (also designated as *Listonella anguillarum*). Outbreaks of this bacterium have been reported for nearly 50 species of fresh- and seawater species in several countries around the world (Thompson, 2006).

Vibrios also infect several aquatic invertebrate animals, such as shrimps, prawn and bivalves. Luminous vibriosis is the term describing the disease of penaeid prawn caused by the bioluminescent *V. harveyi*. Affected larvae of penaeid shrimp develope luminescence reduced feeding, and exhibit poor development. The degeneration of hepatopancreatic tissue results in the formation of necrotic bundles, and increased mortality (Robertson *et al.*, 1998). These infections are usually septicaemic; it has also been suggested that exotoxins may be involved in the process (Harris and Owens, 1999). Bivalve vibriosis is the most commonly encountered disease associated with intensive bivalve

culture in hatcheries and nurseries. Infections are initiated by the attachment of bacteria to the external shell surface along the peripheral valvular margin. Attached bacteria form colonies that grow and contact the mantle, resulting in necrosis of mantle epithelium and penetration of the bacteria into all soft tissues via the coelomic cavity. During the process, the branchial epithelium may also be infected (Elston *et al.*, 1999). Systemic infection of the soft-tissues of the larvae and juveniles (spat or seed) results in tissue necrosis due to production of exotoxin/es by the bacteria, and death. The signs of infection include the sudden onset, with affected larvae exhibiting reduced feeding rate, and erratic swimming behaviour possibly due to the toxin-induced velar damage caused by the bacteria.

Coral bleaching is the major cause of coral reef destruction and has more than one etiology. The loss of colour is the result of the loss of the *Zooxanthellae* (endosymbiotic algae of the coral animal) or the loss of the pigments associated with the algae. At the end of the 90's, the Koch's postulates were applied with success to demonstrate that *V. shiloi* is the causative agent of the bleaching of *Oculina patagonica* (Kushmaro *et al.*, 1996; 1997). Since then, other vibrios have been associated with coral diseases.

Table 3. Non human infection caused by vibrios.

HOST	VIBRIO ASSOCITED	NATURAL DISEASE
Atlantic salmon (*Salmo salar*)	*V. salmonicida*	Cold vibriosis
Coho salmon (*Oncorhynchus kisutch*) Chinook salmon (*O. tshawytscha*) other salmonid fishs	*V. ordalii*	Vibriosis

HOST	VIBRIO ASSOCITED	NATURAL DISEASE
Salmonid fish	*V. anguillarum*	Vibriosis
Eel (*Anguilla anguilla* and *Anguilla japonica*) Shrimps	*V. vulnificus*	Warm water vibriosis
Sea bream (*Sparus aureata*) Cobia (*Rachycentron canadum*) Grouper (*Epinephelus malabaricus*)	*V. alginolyticus*	Septicaemia, exophthalmia and corneal opaqueness
Ayu (Plecoglossus altivelis) Goldfish (Carassius auratus)	*V. cholerae* (non-O1)	Petechial haemorrhages, congestion of the organs
Turbot (Scophthalmus maximus) Sea bream	*V. fischeri*	Neoplasia (visceral tumour), skin papillomas, hemorrhagic ulcerations
Japanese flounders (Paralichthys olivaceus)	*V. ichthyoenteri*	Opaque intestines and intestinal necrosis
Atlantic salmon	*V. logei*	Skin lesson
Iberian toothcarp (Aphanius iberus)	*V. parahaemolyticus*	External haemorrhages and tail rot
Turbot	*V. pelagius*	Eroded fins and tail, internal haemorrhages

HOST	VIBRIO ASSOCITED	NATURAL DISEASE
Turbot Seahorse (*Hippocampus spp.*) Barramundi (*Lates calcarifer*) Common snook (*Centropomus undecimalis*)	*V. harveyi*	Septicaemia Skin infection (ulcerative in some cases) Corneal lesson
Cod (*Gadus morhua*) Turbot Sea bream	*V. splendidus*-related strains	Hemorrhagic areas in the mouth
Coral (*Scleractina order + zooxanthellae*)	*V. coralliitycus* *V. shilonii* (known as *V. mediterranei*)	Coral bleaching and patchy necrosis
Caribbean coral	*V. algynolyticus*	Yellow bloth
Gorgonian coral	*V. harveyi*	Possible surface fouling
Pearl oyster (*Pinctada maxima*)	*V. harveyi*	Tissue lesson
Japanese oyster (*Crassostrea gigas)* Clams (*Ruditapes phillippinarum*)	*V. splendidus*-related strains	Necrosis Muscle alteration
Manila clams (*Tapes philippinarum*) Carpet shell calms (*R.decussatus*)	*V. tapetis*	Brown ring disease (brown deposits on the inner surface of the shells and high mortalities)
Oyster and others bivalves	*V. turbiashii*	Larval mortalities and necrosis of cilia and/or vela

HOST	VIBRIO ASSOCITED	NATURAL DISEASE
Prawn (*Peneaus spp.* and *Macrobrachium rosenbergii*) Lobster	*V. harveyi*	Septicaemia Lessons on exoskeleton Luminous Vibriosis
Penaeids (Penaeus monodon and others)	*V. alginolyticus*	White spot
Prawns (*P.japonicus*) Shrimp (*Litopenaeus stylirostris*)	*V. penaeicida*	"syndrome 93" (erratic swimming, lethargy and weakness. Brown spot in gills and lymphoid organs)
Tiger prawn (*P.monodon*)	*V. parahaemolyticus*	High mortality
Artemia	*V. proteolyticus*	Affect the microvilli, epithelial cells and tissues surrounding the gut
Octopus	*V. splendidus*-related strains	Lessons in arms and head mantle Loss of skin
Scallop (*Pecten maxiums*)	*V. pectenicida*	Death of larvae

Adapated from (Thompson, 2006).

Symbiotic associations

As mentioned, the most studied non-pathogenic vibrio/host interaction is the specific colonization of *Euprymna scolopes* (sepiolid squid) by *V. fischeri* cells (Nyholm and McFall-Ngai, 2004; Ruby, 1996). The squid is nocturnal and emerges at dusk to hunt.

During this period the light organ is full of *V. fischeri* and the bioluminescence of this bacterium is used to camouflage the squid from possible predators. At dawn, the host expels near 95% of the light organ bacteria into the surrounding environment. The squid then buries beneath the sand, and during the day the remaining bacteria grow, consequently at night the light organ is full again. This behaviour allows for the maintenance of the light organ with its symbiotic population and also seeds the environment with *V. fischeri* that colonize new hosts. As both bacteria and host could be maintained in the laboratory, the mechanism of the onset of symbiosis was clarified (Nyholm and McFall-Ngai, 2004).

Vibrio vulnificus

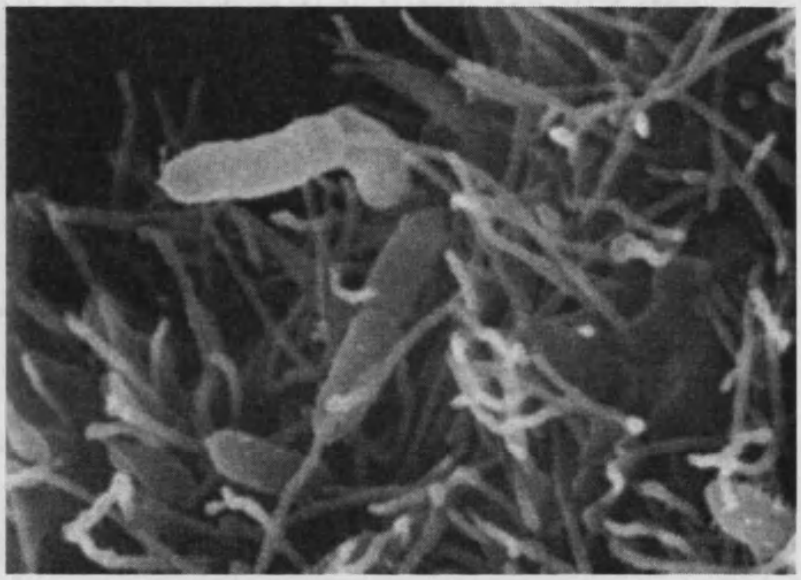
HISTORICAL CONTEXT

In 1976, Hollis *et al.* described a halophilic vibrio, phenotipically near *V. alginolyticus* and *V. parahaemolyticus*, but with the particularity that they were able to ferment lactose, so they were called "Vibrio lactose-positive" or "L+ Vibrio". The same year, Reichelt *et al*, allocate this bacterium inside the genus *Beneckea* under the name of *Beneckea vulnifica* (Reichelt *et al.*, 1976), a name that was included in the "Approved List of Bacterial Names". Later, J.J. Farmer 3rd proposed the transfer of the species into the genus *Vibrio*, and renamed it as *Vibrio vulnificus* (Farmer, 1979).

At the same time in Japan researches described some strains phenotypically and genotypically identical bteween them. Those strains were isolated from diseased eels (*Anguilla japonica*) from six different localizations (Muroga, 1979; Muroga *et al.*, 1976; Nishibuchi and Muroga, 1977, 1980; Nishibuchi *et al.*, 1979; Tison *et al.*, 1982). In 1982, Tison *et al.*, compared those isolates with some clinical and non-clinical isolates of *V. vulnificus*. Based on the results of DNA-DNA hybridization, the authors included the eel isolates into the species *V. vulnificus* (Tison *et al.*, 1982). However, the differences in some phenotypic, serologic and host range

characteristics led the authors to propose the subdivision of the species into two biotypes; biotype 1 would comprise the human and environmental isolates resembling the isolates used in the original description of the species (Reichelt *et al.*, 1976), and biotype 2 those similar to the eel isolates (Tison *et al.*, 1982).

Figure 1. Scanning electron microscopy image of *V. vulnificus* cells.



Source: NOAA/Northwest Fisheries Science Center

More recently, in 1999, (Bisharat *et al.*, 1999; Colodner *et al.*, 2004) a third biotype was described that included strains similar to those isolated in Israel taken from patients with wound infections or bacteraemia who had been in contact with inland-pond-raised tilapia. All those strains showed some biochemical and genetic properties not found in the others biotypes.

SUBDIVISION OF THE SPECIES

V. vulnificus is a highly heterogeneous bacterial species (Arias *et al.*, 1998; Nilsson *et al.*, 2003; Tamplin *et al.*, 1996); as an example, a simple oyster could carry near 1000 different isolates (Buchrieser *et al.*, 1995). The species has been subdivided in biotypes, serotypes and genotypes but none of these classifications

can differentiate human virulent from avirulent strains. In fact, numerous studies have highlighted that no discernible phenotypic differences between virulent and avirulent strains exists since both groups share virulence markers (Jackson *et al.*, 1995).

Serological subdivision

Several serological assays have been used for the identification and subdivision of *V. vulnificus* species. The first serological assay was developed for the species identification and was based on a positive agglutination with *Staphylococcus aureus* Cowan 1 cells coated with rabbit antibodies raised against the flagelar (H) antigen of *V. vulnificus* (Jackson *et al.*, 1997).

The use of serological techniques for the classification of the *V. vulnificus* isolates is based on the lipopolysaccharide (LPS) or O antigen, and three serotyping systems have been proposed. The first was developed by Shimada and Sakazaki, based on agglutination reactions of whole cells with rabbit antibodies raised against heat-killed whole-cell vaccines. This serotyping system subdivided the species in at least seven different serovars but showed significant cross-reactions (Shimada and Sakazaki, 1984). The second serotyping system was proposed by Martin and Siebeling, based on ELISA reactions with monoclonal antibodies raised against purified LPS (Martin and Siebeling, 1991). This scheme subdivided the species in at least five different serovars. However, it was not completely developed and when used in a large collection, it failed mainly in typing environmental strains (Høi *et al.*, 1998a; Zuppardo *et al.*, 2001). The last scheme was developed by Biosca *et al.* for the classification of the eel isolates and it is based on immunoblotting of LPS with polyclonal rabbit antibodies raised against heat-killed whole-cell vaccines. The scheme has shown to be accurate in the subiotyping of biotype 2 and 3 isolates (Biosca, 1994; Biosca *et al.*, 1996b; Bisharat *et al.*, 2007). Two major serovars, serovar E and A, and a minor one,

serovar I (unpublished results), have been described within biotype 2 whereas only one serovar has been found among biotype 3 strains using this system (Biosca *et al.*, 1996b; Bisharat *et al.*, 2007; Fouz *et al.*, 2006). Serovar E is the most virulent for eels and the only one associated with human infections (Amaro and Biosca, 1996). This serovar was used by Tison *et al.* for the original description of the biotype 2 (Tison *et al.*, 1982). Serovar A emerged in 2000 in Spain and is only able to infect fish (Fouz *et al.*, 2007). Unexpectedly, when this polyclonal serotyping system was compared to the one of Martin and Siebeling, the polyclonal sera were able to discriminate three different O-serogroups within the so-called serovar O3, the serogroups A, I and that of biotype 3, suggesting that this system would be more discriminating (unpublished results). Nevertheless, the serology of the species is far from being solved since biotype 1 isolates are the most diverse and they have not been characterized with this polyclonal sera system.

Biotypes

As mentioned, isolates of *V. vulnificus* could be classified into three different biotypes based mainly on differences in biochemical properties, such as indole production and cellobiose fermentation (see table 4), as well as epidemiological pattern and host range (Bisharat *et al.*, 1999; Colodner *et al.*, 2004; Tison *et al.*, 1982). All biotypes have caused infectious diseases in humans, with biotype 1 and biotype 2 strains causing sporadic cases world-wide (Amaro and Biosca, 1996; Dalsgaard *et al.*, 1996b; Gulig *et al.*, 2005; Tison *et al.*, 1982) and biotype 3 strains causing outbreaks in Israel (Colodner *et al.*, 2004). Although all biotypes could be pathogenic for humans, only biotype 2 comprises the strains virulent for fish, mainly eels (Biosca *et al.*, 1991; Dalsgaard *et al.*, 1999; Fouz and Amaro, 2003; Tison *et al.*, 1982).

Table 4. Main differentiate traits of *V. vulnificus* biotypes.

Test[a]	Biotype[b]		
	1	2	3
Ornithine decarboxylase	+	-	+
Indole production	+	-	+
D-mannitol fermentation	+	-	-
D-sorbitol fermentation	+	-	+
Citrate (Simmon's)	+	+	-
Salicin fermentation	+	+	-
Cellobiose fermentation	+	+	-
Lactose fermentation	+	+	-
o-nitrophenyl-b-D-galactopyranoside (ONPG) test	+	+	-

[a]: The differences showed in the table are based on the original description of the biotypes (Bisharat *et al.*, 1999; Tison *et al.*, 1982)
[b]: + = most strains (>75%) positive. - = most strains (>75%) negative.

Biotype 1 is highly heterogeneous and new isolates of biotype 2 have made impossible to use none of these characteristic for the differentiation between biotype 1 and biotype 2 with the exception of the eel virulence. These results would suggest that biotype 2 should be renamed as a pathovar, specifically virulent for eels. Until now, the great majority of the works published concerning the biotype 2 are focused on the serovar E (protagonist also of this dissertation). For the moment, all the biotype 3 strains seem to form a homogenous group confirmed with various studies; they constitute a homogeneous LPS O-serogroup and present a highly conserved nucleotide sequence within genes encoding cell enveloped proteins (Bisharat *et al.*, 2007). In addition, when a wide collection of strains including several biotype 3 isolates were

analysed using a *V. vulnificus* MLST scheme, several sequences types (ST) were obtained but all the biotype 3 strains were included in only one ST (Bisharat *et al.*, 2005).

Clinical *Vs* Environmental isolates

Several studies have focused on finding out any phenotypic/genetic marker differentiating clinical and environmental strains, but until now none of these works have found a useful marker. All the strains of *V. vulnificus* seem to harbour the putative virulence determinants studied until now (DePaola *et al.*, 2003; WHO and FAO, 2005). Warner *et al.*, applied the methodology RAPD-PCR to 31 clinical and 39 environmental strains and found a band of near 200 pb present in 100% of clinical and 7% of environmental strains (Warner and Oliver, 1999). The band was sequenced and although it is not directly involved in virulence, a PCR assay was designed to differentiate C-type (clinical) from E-type (environmental) strains directly from food samples (mainly seafood) (Rosche *et al.*, 2005). When this PCR assay was applied, the authors found that 90% of the isolates classified as C-type were clinical isolates, while 93% of classified E-type were environmental. They also found that 7 of the 25 clinical isolates were E-type, indicating that some patients are more susceptible or that inside the E-genotype exists a subgroup with higher virulence (Rosche *et al.*, 2005).

Examination of the *V. vulnificus* 16S rRNA reveals that at least two sequence variants exist (type A and B). The differences were observed after the alignment of the 16S rRNA sequences of some strains, which showed an area, near the helix 10 of the secondary structure, with higher polymorphism. In a survey of the 16S rRNA genotype, the majority of clinical strains were type B while type A was found in the environmental strains. These results indicate a possible association between type B genotype and human virulence (Aznar *et al.*, 1994; Nilsson *et al.*, 2003).

In a recent study, the genetic relationships between a *V. vulnificus* strain collection were determined using rep-PCR. This technique is based on the amplification of conserved repetitive elements that are distributed throughout the genome and allows strain discrimination with reproducibility. In the same study, the DNA polymorphisms at multiple loci related with the virulence (16S rRNA genotype, C/E profile, and the capsular polysaccharide (CPS) allele) were also determined. A specific "clinical" profile could be identified using rep-PCR that is in agreement with the other loci markers used in the study. The authors indicated that rep-PCR could be a sensitive technique for the determination of the virulence potential of environmental reservoirs (Chatzidaki-Livanis *et al.*, 2006).

ECOLOGY OF *V. vulnificus*

V. vulnificus is a bacterium that occurs naturally in estuaries in many parts of the world. Its distribution and abundance are affected by temperature and salinity of the water. *V. vulnificus* is present in waters, sediments, plankton, molluscs, crustaceans and finfish (Arias *et al.*, 1999; DePaola *et al.*, 1994; Marco-Noales *et al.*, 1999; Oliver *et al.*, 1983; Tamplin and Capers, 1992) and has been isolated from waters with temperatures ranging from 7ºC to 31ºC and salinity between 1-35‰ (Arias *et al.*, 1999; Eiler *et al.*, 2006; Høi *et al.*, 1998b; Wright *et al.*, 1996). Additional factors may also affect the distribution of *V. vulnificus* in the environment, such as nutrient limitation, sunlight, and presence of lytic bacteriophages or competing bacterial population (Marco-Noales *et al.*, 2004). *V. vulnificus* specific phages have been reported in samples from the Gulf of Mexico (DePaola *et al.*, 1997 and 1998); these phages were able to lysate *V. vulnificus* isolates from diseased eels from Denmark (Høi *et al.*, 1998b). In artificial microcosm the cultivability of *V. vulnificus* biotype 2 serovar E was dramatically reduced by the presence of *Aeromonas hydrophila* or *V. vulnificus* biotype 1 cells, suggesting some kind of bacterial competition. This

effect could control *V. vulnificus* population in the natural ecosystem (Marco-Noales *et al.*, 2004).

Starvation and 'viable but nonculturable state'

Bacterial populations in the environment are frequently exposed to stress due to limitation and changes in temperature, nutrient availability, salinity, oxygen saturation and sunlight. Subsequently these bacteria must survive periods of harsh conditions, such as nutrient limitation or temperature extremes. To face these periods, the bacteria have evolved specialized metabolic states that allow them to survive the stressful conditions and still be viable. In general, the microorganism responds to nutrient deprivation by carrying out starvation-induced activities that may include production of degradatives enzymes, as lipases and proteases, and substrate-capturing enzymes, such as alkaline phosphatase. In addition, starved bacteria may differentiate into a more resistant state to maintain the viability (Siegele and Kolter, 1992). One of the most important strategies that are shared by several bacterial species is the entry into "dormant" state, known as viable but nonculturable state (VBNC). Cells in the VBNC state are considered to be metabolically active and therefore capable of resuscitation and regrowth. In *V. vulnificus* the temperature downshift induces the bacteria to enter into the VBNC state in experimental microcosms, and when the temperature is elevated the bacteria recover culturability (Marco-Noales *et al.*, 1999; Oliver, 1995). So, it has been suggested that temperature is an important factor that induces the nonculturable response in natural habitats, and therefore explains why *V. vulnificus* could be isolated almost ubiquitously from water column and sediment in the warmer months, but is undetectable in the cooler monthes. (Oliver and Bockian, 1995). Entrance in the VBNC state involves a variety of morphological and biochemical changes in the cell (Day and Oliver, 2004; Smith and Oliver, 2006), however, the genetic basis and the regulation of the VBNC state remains unclear (Oliver, 2005a).

Biofilm

Bacterial attachment to environmental surfaces and aquatic organism and the formation of multicelullar structures (biofilms) have been shown to be critical for the survival of the genus *Vibrio* (Hood and Winter, 1997; Joseph and Wright, 2004). Biofilms, which provide a protective environment and stability in a changing ambient, are structured communities of adherent microorganisms surrounded by a matrix. This matrix is composed of a mixture of components, such as exopolysaccharide (EPS), protein, nucleic acid, and other substances (Davey and O'Toole G, 2000). *V. vulnificus* is able to produce biofilm on different surfaces. In the case of *V. vulnificus* biotype 2, the biofilm has been suggested as a survival strategy between outbreaks, as it could be detected on the body surface of survival eels that act as carriers (Marco-Noales *et al.*, 2001). The biofilm formation in *V. vulnificus* is inhibited by the glucose (Marco-Noales *et al.*, 2001) and by the CPS that also inhibits the attachment to the surface (Joseph and Wright, 2004). As in other bacteria, the motility mediated by the flagellum and also the type IV pili are necessary not only for the biofilm formation but also for the adherence to biotic or abiotic surfaces (Lee *et al.*, 2004a; Paranjpye *et al.*, 1998; Paranjpye and Strom, 2005). Recently, some genetic elements required for the biofilm formation have been described using a collection of mutant strains derived from random insertion of mini-Tn5 lacZ1. They include proteins involved in transport, enzymes of some metabolic process, protein involved in the stress response, motility, and some regulatory proteins (Kim *et al.*, 2007).

Cell-Cell communication

The biofilm is an example of a collective behaviour of the bacteria since the production and coordination of this community require the bacteria to communicate with each other. As environmental conditions change rapidly, bacteria need to respond

quickly in order to survive. It is very important for pathogenic bacteria during infection of a host to coordinate their virulence in order to escape the immune response and to be able to establish a successful infection. The term "Quorum sensing" (QS) describes the ability of a microorganism to perceive and respond to microbial population density. QS enables bacteria to coordinate their behaviour, usually relying on the production and response to diffusible signal molecules called autoinducer (AI). The AI freely diffuse through the bacterial cell envelops and then, the receptor binds its cognitive autoinducer. When there is sufficiently high concentration of the AI due to high cell density, the complex AI+receptor induces specific gene expression (McDougald *et al.*, 2003; http://www.nottingham.ac.uk/quorum/). Many of the phenotypes regulated by these signalling systems are involved in colonisation and/or pathogenicity and are an advantage only when there is a high cell density.

In the natural environment, there are many different bacteria living together that may use various classes of signalling molecules. The first QS system described was that of *V. fischeri*, this utilizes the production and secretion of N-acylated homoserine lactone (AHL) molecules to regulate the bioluminescence. *V. haveyi* uses two different autoinducer signals. The specie possesses the AHL-mediated system, called signal system 1, composed of an AI synthase (LuxM) and a sensor 1 (LuxN) (Bruhn *et al.*, 2005; McDougald *et al.*, 2003; Milton, 2006). The second system consists of the AI-2 autoinducer, a furanosyl borate diester, which synthesis is dependent on the LuxS enzyme; the periplasmatic protein LuxP binds the AI-2 and initiates the transduction of the signal (McDougald *et al.*, 2003; Milton, 2006). Signals from the sensors converge at an integrator phosphorrelay protein, LuxU, and are finally relayed to a response regulator, LuxO. LuxO is an σ^{54}-dependent transcriptional activator that presumably induces the expression of a repressor of the luciferase structural operon *luxCDABE* (Milton, 2006). *V. vulnificus* was demonstrated to posses the AI-2 system when a *luxR* homologue, *smcR*, was identified and cloned (McDougald *et al.*, 2000, 2001). Looking at

the *V. vulnificus* genome, homologues to the *V. harveyi* LuxS/PQ system have also been found (Chen *et al.*, 2003; Kim *et al.*, 2003a), but no homologues of AHL synthases or AHL signalling molecules have been detected (Milton, 2006). The LuxS quorum-sensing system seems to play an important role in co-ordinating the expression of *V. vulnificus* virulence factors (Kim *et al.*, 2003a).

INFECTION BY *V. vulnificus*

V. vulnificus was named from the Latin word 'vulnificus', which means "inflicting wound(s)", due to the propensity of the organism to cause severe wound infection, cellulitis and sepsis. The first clinical description of *V. vulnificus* was published by Hippocrates (460-377 B.C.) in Epidemics I (see box. I), or at least that is what the historians hypothesised as the symptoms were similar to those described in patients with *V. vulnificus* septicaemia (Kirk and Eli, 2005).

Box 1. First *V. vulnificus* case?

> **Case IX:** Criton in Thasos had a violent pain in the foot which came while walking; it started from the big toe. The same day he took bed with shivering, nausea and slight fever; at night he became delirious. Second day: the whole foot became swollen; it was reddish about the ankle where there was some contraction and small black blisters appeared. He developed high fever and madness. He passed rather frequent unmixed bilious stools. He died on the second day from the beginning of his illness.

V. vulnificus illness has one of the highest mortality rates of any foodborne disease and has emerged as a food safety issue in several countries and regions including Europe, Japan, New Zealand, Republic of Korea and USA (WHO and FAO, 2005). *V. vulnificus* can infect humans via wound exposure or seafood consumption. These infections are rare and generally associated

with underlying conditions. Liver disease, including hepatitis or cirrhosis due to alcohol consumption is an important risk factor. Additional factors include diabetes, gastrointestinal disorders, haematological conditions (anaemia, thrombocytopenia, and leukaemia), and immunodeficiency (Shapiro *et al.*, 1998; Strom and Paranjpye, 2000). The *V. vulnificus* infections are usually produced as sporadic cases associated with raw seafood ingestion or wound infections associated with seawater. The only exception is the biotype 3, which produced outbreaks associated with the manipulation of spiny cultured fish (Tilapia). The clinical manifestations are exactly the same regardless of biotype. To date, only biotype 1 has been implied in food-borne infections whereas biotype 2 is the only one recognized as a zoonotic agent (Amaro and Biosca, 1996; Dalsgaard *et al.*, 1996b).

Clinical manifestation

Infection in humans

Case reports of *V. vulnificus* infection have been described in several countries throughout the world. Because surveillance of *V. vulnificus* infections is poor, the number of cases reported is likely to be underestimated. In a recent study, 386 physicians in Japan were questioned about *V. vulnificus* infection. More than 85% of responders stated they had no knowledge about the bacteria. The author of this study estimated the annual number of cases of *V. vulnificus* septicaemia as 425, around three time the number of cases described in USA or Korea (Osaka *et al.*, 2004).

V. vulnificus is unique in its ability to cause three distinct syndromes of infection. Out of 422 infections reported to the CDC between 1988 and 1996, 45% were wound infections, 43% primary septicaemia and 5% gastroenteritis (7% were from undetermined exposure) (Shapiro *et al.*, 1998). In a review of 134 cases published between 1980 and 1995 in Asia, only 0.8% presented

gastroenteritis, 79% were primary septicaemia and 18% wound infections (Chan, 1995).

Gastroenteritis

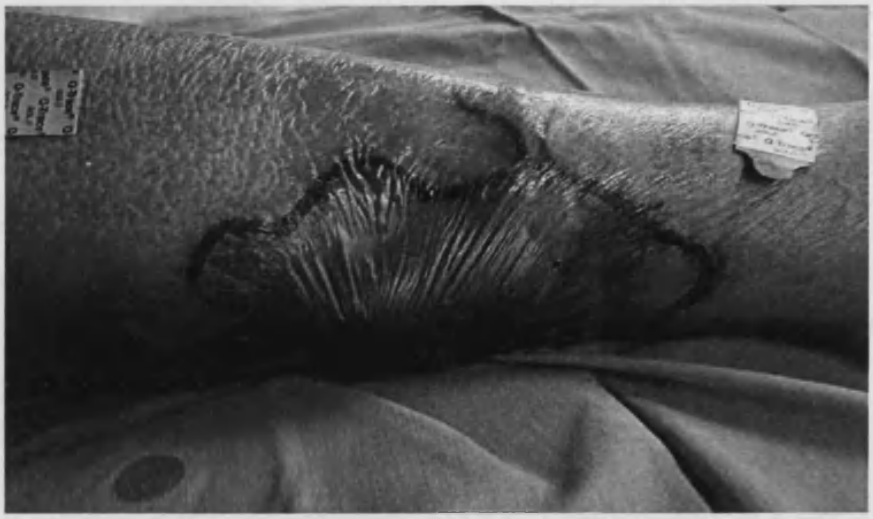
The majority of the *V. vulnificus* associated gastroenteritis cases appear to be self-limiting and probably go largely unreported (Strom and Paranjpye, 2000). In fact the CDC estimates to have a 20:1 under-reporting ratio (WHO and FAO, 2005). Almost always the infection follows the ingestion of raw seafood. In the most severe cases, symptoms include fever, diarrhoea, abdominal cramps, nausea, and vomiting and hospitalization is required. *V. vulnificus* is isolated from stool specimens. However, the relationship between *V. vulnificus* and gastroenteritis is not yet clear, as other possible causes have not been investigated (Strom and Paranjpye, 2000).

Primary septicaemia

The most significant form of *V. vulnificus* disease is a primary septicaemia, and is defined as a systemic illness characterized by fever or shock (systolic blood pressures <90 mm Hg) in which *V. vulnificus* is isolated from either blood or a normally sterile site and no wound infection preceded the illness. Primary septicaemia follows ingestion of raw or undercooked seafood, mainly oysters and occurs in person with certain underlying and chronic diseases (Shapiro *et al.*, 1998; Strom and Paranjpye, 2000). In most cases, these pre-existing disorders result in an elevation of serum iron, which has been shown to increase the ability of *V. vulnificus* to survive and grow in human serum (Weinberg, 2000). Patients with primary septicaemia usually exhibit fever and chills, sometimes accompanied by vomiting, diarrhoea and abdominal pain. Quite often in a *V. vulnificus* septicaemia secondary skin lesions appear on the extremities, these unusual bullae become large and filled with hemorrhagic fluid, and may form necrotic ulcers (necrotizing fasciitis) or even gangrenous,

requiring tissue debridement or amputation (Gulig *et al.*, 2005; Oliver, 2005b).

Figure 2. *V. vulnificus* infection in humans. Blistering cellulitis and necrotising fasciitis in a leg.



Source:(Ralph and Currie, 2007)

Wound infection

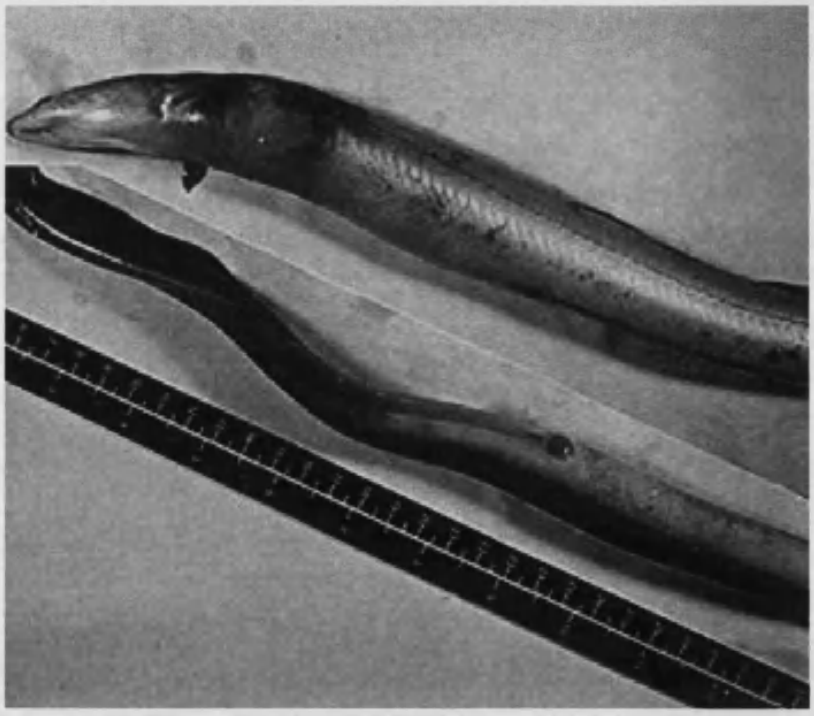
Wound infections typically result from either injury to the skin in a marine environment or while handling finfish or seafood, or contact of a pre-existing wound with seawater. Patients often present with fever, pain and swelling at the wound site. The skin and soft tissue infection can rapidly progress from erythema to cellulitis to necrotizing fasciitis. Often the infection can progress to septicaemia and presents symptoms similar to primary septicaemia (Gulig *et al.*, 2005; Oliver, 2005b).

Vibriosis in fish

V. vulnificus could affect several species of aquatic animals but is the eel vibriosis the disease economically most important. The eel vibriosis appears as epizootics or outbreaks of high mortality affecting cultured eels both in seawater and freshwater worldwide. The clinical symptoms are similar to those described for

human vibriosis since the disease in its acute form is a primary septicaemia characterized by external and internal haemorrhages affecting the main body organs such as liver, kidney, spleen, and pancreas (Biosca *et al.*, 1991). The first isolates of *V. vulnificus* biotype 2 were recovered from Japanese eels (*A. japonica*) between 1975 and 1977 (Muroga, 1979; Muroga *et al.*, 1976). In 1989, the vibriosis was first described in Europe, where recurrent outbreaks affected cultured European eels in Spain (Biosca *et al.*, 1991). The disease has spread to the rest of Europe affecting the eel production in Denmark, Norway, Sweden, and England (Austin and Austin, 1999).

Figure 3. Vibriosis in eels. The eels presented classical external symtoms as haemorragies in fins, ulcers and petequias.

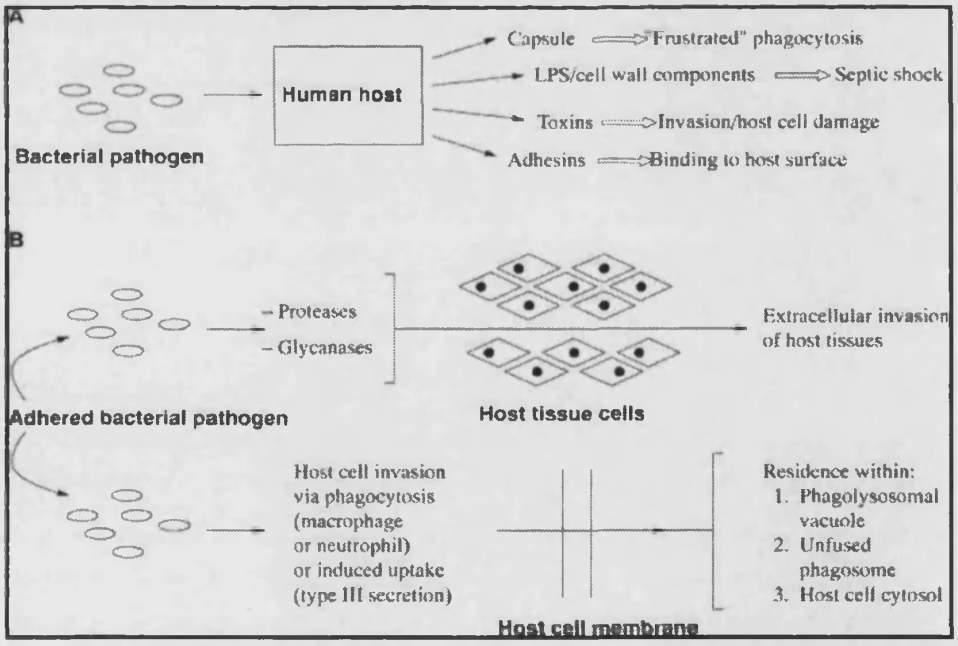


Source: (Amaro *et al.*, 2001)

VIRULENCE FACTORS

Although a significant number of studies concerning virulence factors of *V. vulnificus* have been published, nothing remains clear and the virulence that seems to be multifaceted is not well understood. Production of disease by this bacterium involves a wide range of virulence factors together with host susceptibility. Construction of specific mutations in genes controlling these virulence factors have shown that only the capsule, the presence of a functional flagellar biogenesis system, and the siderophore to acquire iron from the transferrin are essential for virulence (Litwin *et al.*, 1996; Simpson *et al.*, 1987). The virulence determinants have been found in all the strains, regardless the origin (clinical or environmental) or the biotype of the strain. The studies done with serovar E reveal a similar virulence pattern with biotype 1 and the same could be expected for the biotype 3, even though no studies are published about its virulence factors.

Figure 4. An overview of bacterial mechanisms for pathogenicity.



Source: (Wilson *et al.*, 2002)

Capsular polysaccharide

The capsular polysaccharide (CPS) of *V. vulnificus* has been defined as its most important virulence factor (Biosca *et al.*, 1993b; Gulig *et al.*, 2005; Simpson *et al.*, 1987). Encapsulated isolates have an opaque colonial morphology (Op), while the bacteria that grow forming a translucent colony (Tr) have a decreased amount of CPS. Expression of CPS is correlated with lethality in mice and eels (Amaro *et al.*, 1994; Simpson *et al.*, 1987), cytokine production (Powell *et al.*, 1997), and resistance to phagocytosis and opsonization (Shinoda *et al.*, 1987; Tamplin *et al.*, 1985). *V. vulnificus* presents a phase variation from opaque to translucent morphology with a high frequency (Grau *et al.*, 2005; Hilton *et al.*, 2006; Wright *et al.*, 2001) and the reverse switch has also been reported with lower frequency (Simpson *et al.*, 1987). CPS operons in *Escherichia coli* are classified into groups 1 to 4 based on CPS biochemical and genetic organization (Whitfield and Roberts, 1999). One *V. vulnificus* CPS operon has been described and shows high homology to the conserved group 1 of the *E. coli* (Wright *et al.*, 2001). Most of the encapsulated strains (88%) of *V. vulnificus* are classified into group 1, however different capsular types have been found among environmental and clinical isolates (Hayat *et al.*, 1993) suggesting that *V. vulnificus* has different metabolic pathways for the biosynthesis of the CPS (Chen *et al.*, 2003).

More recently a third phenotype has been described; the rugose colony. This phenotype was isolated from the opaque colony and, interestingly, it was highly resistant to the complement of the human serum (Grau *et al.*, 2005).

In the case of biotype 2, the capsule improves the bacterium capability for colonising the host. Thus, translucent strains are avirulent when infecting through the water pathway, whereas they are virulent for eels by intraperitoneal injection (Amaro *et al.*, 1995; Amaro *et al.*, 1994; Biosca *et al.*, 1993b).

Lipopolysaccharide

The symptoms observed in the septicaemia due to *V. vulnificus* and the inflammatory response in the wound infections could be associated to the endotoxic activity of the lipopolysaccharide (LPS). McPherson *et al.* (1991) suggested that *V. vulnificus* LPS may be a factor contributing to the virulence. They showed that LPS was pyrogenic by intraperitoneal injections and decreased the mean arterial pressure within 10 minutes with further declined leading to death in 30 to 60 minutes. Further, it was demonstrated that purified LPS caused an increase in inflammation-associated cytokines release (IL-6 and TNF-α) from human peripheral blood monoclonal cells (PBMCs), although the response was less than that measured using purified CPS (Powell *et al.*, 1997).

The diseases caused by *V. vulnificus* in humans present a significant difference in the rates of primary septicaemia between men and women. Around 80% of the cases compiled by the FDA in USA are men. (Shapiro *et al.*, 1998). Merkel *et al* (2001) demonstrated, using a rat model, that the estrogen protects against the *V. vulnificus* LPS-induced endotoxic shock.

In the case of biotype 2 and eel virulence, it has been suggested that LPS could be responsible for host specificity because it confers resistance to eel complement activated by the alternative pathway. In eels, the LPS could be responsible for host specificity since it is the main surface component that discriminated eel-virulent from eel-avirulent *V. vulnificus* strains (Amaro and Biosca, 1996; Amaro *et al.*, 1992a). In this way, biotype 1 strains would be destroyed by eel complement whereas biotype 2 strains would be resistant and multiply. Amaro *et al.* (1997) demonstrated that the O side chain of the LPS (of the serovar E) act as a protective factor against the non-specific immune response. The O-antigen of the serovar E appears to determine the resitance to the bactericidal effect of the non-immune eel serum, which destroy biotype 1

strains and also a spontaneous rugose mutant of biotype 2 lacking this LPS component (Amaro *et al.*, 1997).

Adhesins

A first step in the disease progress caused by extracellular pathogens is the successful colonization of host tissues by means of adhesins (surface bacterial proteins) that specifically bind eukaryotic receptors on host tissue cells. Many adhesins are located on the tip of fimbriae or pili (fimbrial adhesins) whereas others are surface proteins (non-fimbrial adhesins). Few studies are published concerning adhesin production by *V. vulnificus*, Gander and LaRocco (1989) described the presence of a "pilus-like structure" that was correlated with the adherence to monolayers of Hep-2 cells. Those structures were closely associated with the origin of the isolate, clinical or environmental one. Later, the laboratory of Dr. Mark Strom identified the genes encoding proteins required for the pili biogenesis. The genes form a cluster *pilABCD* closely related to the one described in other gram-negative bacteria as *V. cholerae* (Paranjpye *et al.*, 1998; Paranjpye and Strom, 2005). A mutation on *pilD* (a peptidase/N-methyletransferase protein) caused a broad pleitropic defect that prevents expression of pili on the surface. In addition, the mutation blocked extracellular secretion of several proteins that utilize the type II secretion pathway, reduce the cytotoxic activity, adherence to cultured cells, and increased the LD_{50} in iron-dextran treated mice (Paranjpye *et al.*, 1998). Mutation in pilA (the major pilin protein) reduces the biofilm formation and significantly decreases adherence to Hep-2 cells and virulence in iron dextran treated mice (Paranjpye and Strom, 2005).

Flagella

As are other components of the genus *Vibrio*, *V. vulnificus* is a motile bacterium, in this case, by means of a single polar flagellum. The motility of bacterial cells is supposed to be

important for pathogenic bacteria, necessary for penetrating mucosa and gaining access to the underlying epithelial cells (Lee *et al.*, 2004a; Ran Kim and Haeng Rhee, 2003). By means of the generation of mutants defective in genes of the flagelar hook-basal body that were non-motile, two different groups demonstrated the role of the flagellum in virulence (Lee *et al.*, 2004a; Ran Kim and Haeng Rhee, 2003). Mutants in the genes *flgC* (flagella basal body rod protein) and *flgE* (flagellar hook protein) were non-motile but also showed a significant decrease in adhesion, cytotoxicity, and lethality to mice.

One of the structural components of the flagella filament of *V. vulnificus*, the flagellin, FlaB, has been proposed as a mucosal adjuvant. The coadministration of FlaB with tetanus toxoid (TT) significantly enhances TT-specific immunoglobulin (IgA and IgG) response in the intranasal immunization mouse model. The result of this study indicates that the flagellin would be an efficacious mucosal adjuvant inducing protective immune response through Toll-like receptor 5 (TLR5) activation (Lee *et al.*, 2006).

Iron utilization

Iron has an essential role in many diverse biological systems and the requirement of iron is extended to nearly all living beings. It is essential as cofactor for several enzymes and participates in the oxygen metabolism. However, because of the low solubility of ferric iron and its potential toxicity, higher organisms have evolved mechanism for lowering the level of free iron. In mammals and fish the majority of the iron is intracellular and complexed with proteins such as haemoglobin, transferrin, cytochrome C or stored in the iron-storage protein, ferritin. Bacteria also need iron for growth and therefore, pathogens have evolve efficient iron acquisition strategies, including reduction of ferric to ferrous iron, occupation of internal niches, utilization of host iron compounds, and production of siderophores (Wooldridge and Williams, 1993). Iron and iron-acquisition systems seem to be important for the

virulence of *V. vulnificus* because of the correlation between increased iron levels in the host, and susceptibility to infection (Gulig *et al.*, 2005; Wright *et al.*, 1981). *V. vulnificus* can obtain iron for growth by using different strategies; i) producing and secreting siderophores to compete for iron with the iron-chelating proteins of host (transferrin and lactoferrin) and ii) by binding heme group of haemoglobin and other haemin compounds by means of specific receptors. Although *V. vulnificus* biotype 1 can produce two types of siderophores (Simpson and Oliver, 1983), only the phenolate-type (vulnibactin) has been chemically characterized. Vulnibactin production has been related to human virulence since deficient mutants showed reduced virulence in mice. Recently, the implication of vulnibactin in iron-uptake from human transferrin has been demonstrated (Sun *et al.*, 2006). In addition, *V. vulnificus* can use heterologous siderophores like ferrioxamine B, which could be secreted by members of the gut commensal flora (Aso *et al.*, 2002).

V. vulnificus biotype 2 produces hydroxamates and phenolates (Biosca *et al.*, 1996a) as well as binds hemin (Fouz *et al.*, 1996). In contrast to biotype 1, when bacteria were grown in a medium with ferric transferrin as sole iron source, only hydroxamate type siderophores were produced, which suggests that hydroxamates may have a role in fish virulence. Therefore, this bacterium has evolved a series of strategies to acquire iron inside the mammalian host as well as teleost fish and shellfish.

Haemolysins

Haemolysins are pore-forming toxins that have in common their ability to lysate erythrocytes producing the liberation of the haemoglobin (Cossart *et al.*, 2005). They are the most widely distributed toxin among pathogenic vibrios and exercise various roles in the infectious diseases. Sometimes, the lytic activity is extended to other cellular types, like in the case of *V. vulnificus* haemolysin/cytolysin (VvhA) (Lee *et al.*, 2004b). The lysis of the

cells by this enzyme is due to the formation of small pores in the membrane by cholesterol-mediated oligomerization of the protein (Kim and Kim, 2002; Kim *et al.*, 1993). In animal models, the injection of purified VvhA reproduces the pathological effects of the injection of live bacteria (Gray and Kreger, 1985, 1987; Park *et al.*, 1996). This enzyme shows a high binding affinity to cell membranes of a wide range of eukaryotic cells such as neutrophills, mast cells, endothelial cells, and macrophages (Kang *et al.*, 2002; Kim *et al.*, 1993; Kim *et al.*, 1998; Kwon *et al.*, 2001; Park *et al.*, 1994). VvhA is also able to stimulate the production of signalling molecules such as hydrogen peroxidase (H_2O_2), which activate the apoptosis (Cossart *et al.*, 2005), and nitric oxide (NO), which contributes to host-induced tissue damage causing hypotension and septic shock. Despite these effects, VvhA seems not to be very important in virulence for mice, since *vvhA* mutants show the same LD_{50} than the wild type (Fan *et al.*, 2001; Wright and Morris, 1991).

Two other haemolysins have been described in *V. vulnificus*; the deduced amino acid sequence of the first one (VllY) present a high homology with the legiolysin from *Legionella pneumophila*, a protein implicated in haemolysis, pigment production and fluorescence. The role of the VllY in virulence was not studied (Chang *et al.*, 1997). The third haemolysin described (HlyIII) has a nucleotide sequence similar to haemolysin III of *Bacillus cereus*. This haemolysin seems to be implicated in virulence since *hlyIII* isogenic mutants showed attenuation in virulence compared with the wild-type by intraperitoneal injection into mice (Yu-Chung *et al.*, 2004).

Metalloprotease

The *V. vulnificus* metalloprotease (designed Vvp) is an extracellular protease that requires zinc for its catalytic activity (Gulig *et al.*, 2005; Strom and Paranjpye, 2000). The Vvp shows proteolytic activity against a broad-range of substrata such as

collagen, elastin, and casein. It has been proposed that Vvp could be the main virulence factor responsible for the skin lesions, because the purified Vvp enhances vascular permeability that mediates edema and induces serious haemorrhagic damage through digestion of the vascular basement membrane (Miyoshi, 2006). The role of Vvp in facilitating iron acquisition has been proposed by different authors. Furthermore, protease mutant were similar to the wild-type strain, as no attenuation of the mice virulence or cytotoxicity to cell culture were observed (Jeong *et al.*, 2000; Shao and Hor, 2001). The same effect was observed when a double mutant in both *vvP* and *vvhA* was used (Fan *et al.*, 2001) suggesting that none of the enzyme are required for virulence, even thought the cytolisin seemed to be an important cause of damage in the alimentary tract of the mice (Fan *et al.*, 2001).

The fact the haemolysin/cytolysin, metallopreotease or double mutants were still invasive, lethal to mice, and cytotoxic strongly suggests the presence of some unidentified cytotoxin. Therefore, the mutation in one enzyme could be compensated by other/s and the bacteria keep its infectivity. In fact, when the genome of *V. vulnificus* was analysed, 13 open reading frames (ORF's) with significant homology with other cytotoxins or haemolysins were identified (Chen *et al.*, 2003).

Recently, it has been demonstrated that Vvp, in biotype 2, is essential for colonization of mucosal surfaces including fish and algae mucus. Deficient mutants in this protein exhibited reduced chemostasis toward and attachment to mucus and showed significant attenuation in eel virulence by bath infection (Valiente, 2006).

Other virulence factors

A recently identified determinant of virulence in V. vulnificus is a member of the RTX family of toxins produced by a limited group of Gram-negative pathogens. The RTX locus in *V. vulnificus* is on the large chromosome I. It includes four genes (rtxA, rtxC, rtxB and rtxD) encoding the Rtx toxin, toxin activator, and associated transport system. Additional RTX-like molecules have been found in the genome of *V. vulnificus* (Chen *et al.*, 2003). RTX toxins cause pore formation in red blood cells, necrotic death of Hep2 cells, and depolymerization of actin in HeLa cells (Gulig *et al.*, 2005).

V. vulnificus exports a number of extracelllular enzymes (apart from the ones cited before) like mucinase, lipase, chondroitinase, hyaluronidase, DNase, esterase, and sulfatase (Gulig *et al.*, 2005). These degradatives enzymes could be considered virulence factors that contribute to colonization and invasiviness, however, the real role in virulence remains unknown (Strom and Paranjpye, 2000).

Virulence regulation

The studies conducted to understand the regulation of virulence gene expression in *V. vulnificus* are based on well characterized bacterial regulatory systems (Gulig *et al.*, 2005). One example is the expression of the iron-uptake systems depending on vulnibactin and heme-compounds controlled by a Fur repressor (ferric uptake regulator) which poses high homology to the one described in *V. cholerae* (Litwin and Calderwood, 1993). In *V. vulnificus*, Fur protein regulates the vulnibactin mediated iron-uptake and heme utilization system and therefore has an important task in the virulence (Litwin and Byrne, 1998; Webster and Litwin, 2000).

Catabolite repression mediated by cyclic-Adenosine monophosphate (cAMP) and its receptor protein (CRP) is primarily responsive to carbon availability and regulates the expression of a variety of genes including some virulence factors. Mutations in the gene cya (adenylate ciclase, which is required for cAMP synthesis) or in the receptor crp produce pleiotropic changes that affect capsule, protease and cytolisin production, motility, cytotoxicity and mouse lethality (Kim *et al.*, 2005).

The protein RpoS is an alternative sigma factor, which is important in stress response and virulence (Hulsmann *et al.*, 2003; Richard and Foster, 2003; Rychlik and Barrow, 2005; Venturi, 2003). Mutants in this gene exhibit a decreased ability to survive under environmental stress, including exposure to hydrogen peroxide, hyperosmolarity, and acidic conditions. These mutants also show a decrease protease production and motility (Hulsmann *et al.*, 2003).

Regulation of *V. vulnificus* virulence by the LuxS (AI-2) quorum-sensing system has been demonstrated. The deletion of the gene luxS produces a delay in protease production but an increase of the haemolysin, and attenuation in virulence degree for mice (Kim *et al.*, 2003a). In contrast, mutations in luxR (the regulator that senses the autoinducer) does not reduce virulence degree for mice, although they affect protease and haemolysin production (Shao and Hor, 2001). Recently, Kawase et al reported that the protease gene was regulated by LuxS during growth in LB, but not in serum, where its expression was related to iron levels (Kawase *et al.*, 2004).

Host susceptibility

V. vulnificus is a particularly virulent vibrio, which commonly produces either wound infection or primary septicaemia with a high mortality rate. Septicaemia is associated with various predisposing conditions (Gulig *et al.*, 2005), usually chronic diseases

affecting either liver or immune system functions (Strom and Paranjpye, 2000). It should be noted that *V. vulnificus* infection is a rare event. For example, in USA, the FDA estimates that between 12 to 30 million of people in this country can suffer one of the predisposing conditions but the number of fatal *V. vulnificus* infection reports (around 20 each year in USA) are far fewer than expected (Rosche *et al.*, 2005; WHO and FAO, 2005).

Chronic liver diseases (cirrhosis, hepatitis, alcoholic liver disease, and hepatoma) and hemochromatosis are usually associated with fatal cases. A common characteristic of these syndromes is an elevated amount of iron in blood that favours the growth of the pathogen (Weinberg, 2000). In addition, iron overload decreases antibody-mediated and mitogen-stimulated phagocytosis by monocytes and macrophages, alters T-lymphocyte subsets, and modifies lymphocyte distribution in different compartments of the immune system (Walker and Walker, 2000). Other conditions that predispose people to *V. vulnificus* infection are those that suppress immune system functions, such as AIDS, some cancers, and prolonged therapy with systemic corticosteroids (i.e. asthma, rheumatoid arthritis). Diabetes and renal diseases have been also considered as predisposing factors, since these chronic diseases could affect also the correct function of the immune system (WHO and FAO, 2005). Finally, other putative risk factor is associated to the use of antacids and acid blockers that could substantially increase the chances of gastric survival and influence subsequent infection (Koo *et al.*, 2000; Koo *et al.*, 2001). However, this possibility is controversial (Tacket *et al.*, 1984).

Regarding eels, no predisposing factor seems to exist. In fact, when a epizootic breaks out in a fishfarm for the first time, the vibriosis is fulminant, given that the pathogen is highly virulent for non-immunized animals (Amaro *et al.*, 1995). The vaccination of eels with Vulnivaccine (licensed by the University of Valencia, Spain; concession number 9701300) induces high protection against the vibriosis caused by *V. vulnificus* serovar E for a period of at least 6 months (which is the main growth period of animals). After

this period of time fish could be treated with a vaccine booster orally before stressful periods. These procedures would guarantee the healthy status of eels for human consumption and animal manipulation (preventing zoonosis) (Collado *et al.*, 2000; Esteve-Gassent *et al.*, 2004a; Esteve-Gassent *et al.*, 2004b; Fouz *et al.*, 2001).

MODELS FOR STUDYING *V. vulnificus* INFECTION

Cell culture

Cultured cell lines constitute an important tool for studying microbial pathogenesis. However the use of cultured cells for the analysis of host-pathogen relationships presents important limitations since the results can differ significantly between cell lines, and cell lines are generally grown in stabilized media, whereas in the host tissues, different cell types co-exist and share cross-talk molecules. In conclusion, the use of cell lines can give important information about the virulence of a pathogen, but results must be confirmed in more complex systems like animal models when possible (Hautefort and Hinton, 2002).

Cell cultures have been applied for studying *V. vulnificus* pathogenesis, although most of these works have been focused on the evaluation of the effect of purified toxins and enzymes on selected cell lines. Very few of them focused on adherence or/and invasion of eukaryotic cells (Gulig *et al.*, 2005).

Rodent animals

Rats and mice are the most commonly used animal models to study *V. vulnificus* infection in humans. Sometimes a pre-treatment with some form of iron is used in order to mimic the

predisposing conditions defined as risk factors in humans (Gulig *et al.*, 2005). Subcutaneous inoculation reproduces the *V. vulnificus* wound infection. In fact, the histological damage observed after the inoculation is similar to that described in human infection with the exceptions of bulbous skin lesions that are only observed in humans. Oral inoculation will be the ideal form to reproduce the primary septicaemia. Fan et al (2001) determine that the cytolisin - but not the protease - seem to be important in causing damage in the alimentary tract of the mice when the bacteria are administrated by this route.

Eel

The best model for studying an infection is the natural host, and this is possible in the vibriosis due to *V. vulnificus* biotype 2 in eels. Those fish are equipped with a strong innate immune system protecting them against infections in a changing environment, from marine to brackish and fresh waters (Nielsen and Esteve-Gassent, 2006). *V. vulnificus* uses the gills as main portal of entry (Marco-Noales *et al.*, 2001). From there, bacteria enter the blood almost immediately and spread to internal organs, where the bacteria persist (Valiente and Amaro, 2006). Eels can successfully be vaccinated against the vibriosis and the immunological memory is responsible for a long-term protection (Collado *et al.*, 2000). In the works of the vaccine development and validation, the acquired immune response against *V. vulnificus* was well characterized (Esteve-Gassent and Amaro, 2004; Esteve-Gassent *et al.*, 2004a; Esteve-Gassent *et al.*, 2004b; Esteve-Gassent *et al.*, 2003; Fouz *et al.*, 2001). Those studies suggested the presence of two immune compartments, systemic and mucosal, as different kinetics of antibody production were archived (Esteve-Gassent *et al.*, 2003). Systemic and also mucosal antibodies play an important role in protection, recognizing the pathogen surface antigens as well as bacterial toxins (Esteve-Gassent and Amaro, 2004).

With respect to the bacteria, some virulence factors have been seen to be important for the virulence to eels. In this introduction, it was mentioned the role of the capsule, LPS, or the siderophores, but more works regarding the exotoxins in the eel vibriosis are necessary.

Caenorhabditis elegans

The nematode *C. elegans* is emerging as a facile and economical model host for the study of evolutionarily conserved mechanism of microbial pathogenesis and innate immunity (Sifri *et al.*, 2005). Recently, the feasibility of *C. elegans* as experimental model for *V. vulnificus*-host interaction has been suggested (Dhakal *et al.*, 2006). In this study, virulent strains of *V. vulnificus* were able to infect and kill the worm and an attenuated worm-killing ability was observed when *V. vulnificus* mutants on virulence genes were used for feeding the nematode.

Zebrafish

Teleost fish, like zebrafish (*Danio rerio*) have a well-developed immune system, both innate and adaptative, which is quite similar to the mammalian immune system (Trede *et al.*, 2004; 2001). In addition, its small size, rapid generation time and powerful genetic systems (several genetic tools to study zebrafish have been developed) and genomic resources (the complete genome will be available soon by the Sanger institute) make this fish one of the most versatile animal models for the study infectious diseases (Neely *et al.*, 2002; van der Sar *et al.*, 2003). Furthermore, zebrafish embryos develop externally and are optically transparent allowing the localisation of the pathogen during infection (O'Toole *et al.*, 2004a; van der Sar *et al.*, 2003). The availability of the whole genome sequence will allow the production of numerous mutants affecting organogenesis and development (Zon, 1999). Some of these mutants could mimic the predisposing conditions for

infection, like the ones in *V. vulnificus* infection. Nevertheless, a few bacterial pathogen-zebrafish models have been established (Neely *et al.*, 2002; O'Toole *et al.*, 2004b; Trede *et al.*, 2004; van der Sar *et al.*, 2003) , that for the moment do not included *V.vulnificus*.

2

OBJECTIVES AND WORK PLAN

The present work is included in the research program of the "Pathogen of aquatic animals with Public Health interest" unit of the Microbiology and Ecology Department in the Faculty of Biology of the University of Valencia. This group has been working on different aspects of *V. vulnificus* biology, focusing on the fish pathogen biotype of the species (biotype 2). Since the date of the first isolation of the pathogen in a Spanish eel farm, 1989, different studies have been performed that include basic research (i.e. survival strategies inside and outside the host, induction of the VBNC state, infection routes and portal of entry into the host...) and applied research (diagnostic and identification methodologies, antibiotic resistance, vaccines...). The team has developed a vaccine (Vulnivaccine patented in 2000) and a vaccination procedure that successfully controls the eel vibriosis at farms. Derived from the vaccine development research a complete study of the immune response of the eel to the *V. vulnificus* infection and vaccination procedures was performed. At present, the research is focused in the mechanisms that generate intraspecific variability in the species as well as in the host-parasite interactions under a molecular point of view. Under this scenario the present Ph.D project was posed with the title of "Epidemiology and phylogeny of the pathogen *Vibrio vulnificus* biotype 2".

The main objective of the project was to find out how the eel virulent clones had appeared in the evolutive history of the species *V. vulnificus*. With this purpose, we amplified the genetic variability of our collection of biotype 2 strains by developing and applying specific protocols for its isolation from the environment in absence of epizootic events. Then, we used a series of phenotypic and genotypic methodologies, including partial gene sequentiation, with the whole collection of strains that represents the genetic variability of the whole species in order to study and built the phylogeny of the eel pathogenic group. Finally, the generated knowledge was applied to the development of new methodologies for the rapid identification of the pathogen and carrier detection. The partial objectives were:

1. **Demonstrate that *V. vulnificus* biotype 2 is an aquatic bacterium by its isolation in absence of epizootic events.** Previous work of the unit demonstrated that biotype 2 could survive in artificial and natural seawater microcosm even trough it had not been isolated from water ecosystems. With this aim, some specific objectives were proposed:

   1.1. To develop a new selective biotype 2 specific isolation procedure for water or aquatic organism samples.
   1.2. To validate the new isolation procedure with natural samples for its use in epidemiologic and epizootiology studies.

2. **Design a fast method for biotype 2 identification that allows the discrimination of the zoonotic serovar.** The selected method was a Multiplex PCR assay. For its design we follow the next steps:

   2.1. Find out biotype 2 and serovar E specific DNA sequences by the use of the subtractive hybridization procedure.
   2.2. Test the specificity of these sequences by PCR with a wide collection of *V. vulnificus* isolates of different biotypes and serovars and other related bacteria.
   2.3. Design a Multiplex PCR method for the identification of *V. vulnificus* biotype 2 and discrimination of the serovar E strains.

2.4. Validate the Multplex PCR as a vibriosis diagnostic tool and carrier detection.

3. Study the intraspecific variability and find, if possible, some reliable markers for biotype, serovariety or virulence potential for humans and/or animals. *V. vulnificus* was described as a highly heterogeneous bacterium but few reports have studied its variability including strains of all biotypes. Some specific objectives were performed.

3.1. Collect a wide collection of *V. vulnificus* isolates that include representative strains from different sources and from worldwide origin.
3.2. To characterize the collection using biochemical, physiological (API strips, Biolog GN plates) and molecular methods (riboprinting and polymorphism in different loci).
3.3. To analyze the data obtained to reveal similarities and differences between the groups established.

4. To construct the phylogenetic history of *V. vulnificus* and the clonal complexes of the species. To determine the relationships of the biotype 2 with the other biotypes and draw the evolutionary history of the biotype within the species we perform MLSA analysis that was divided in the following tasks:

4.1. Sequence internal fragment of some housekeeping genes jointly with other virulence related genes in all the *V. vulnificus* collection.
4.2. Perform the analysis of the sequences: Editing, alignment of the different genes and construction of phylogenies. The clonal structure of the strains studied was also determined with specific bioinformatics programs.

3

A PROTOCOL FOR THE SPECIFIC ISOLATION OF VIRULENT STRAINS OF *V. vulnificus* SEROVAR E (BIOTYPE 2) FROM ENVIRONMENTAL SAMPLES

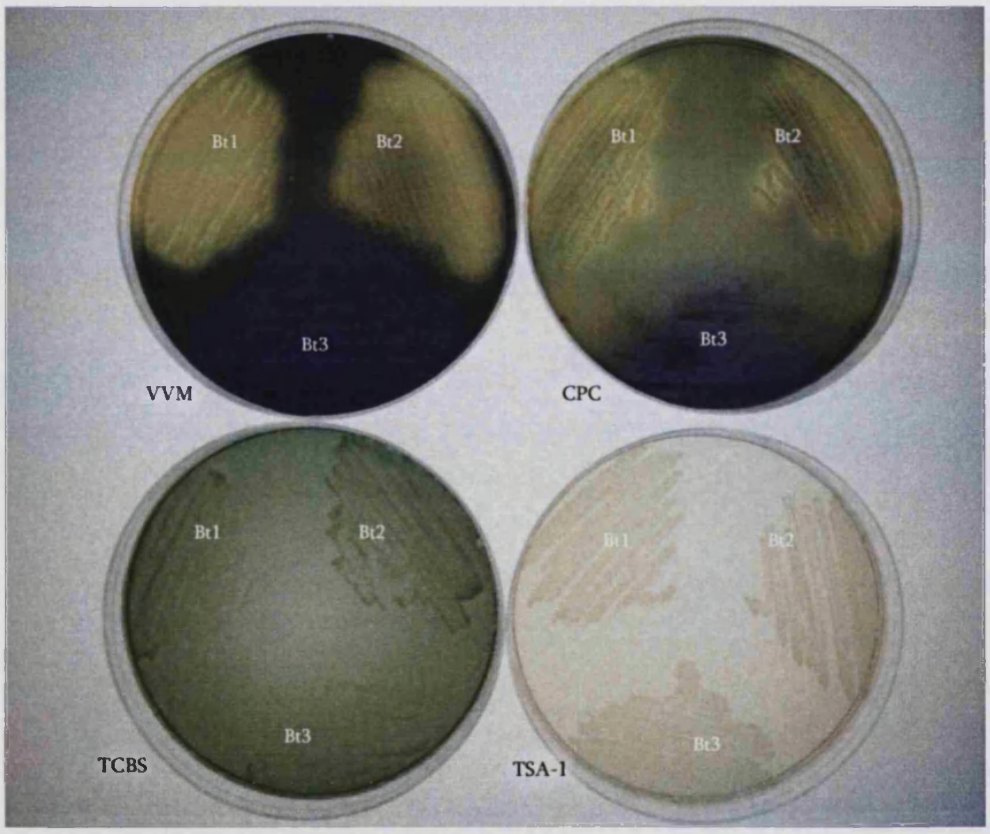
INTRODUCTION

V. vulnificus biotype 2 was initially described as an obligate eel pathogen in spite of the species is autochthonous from aquatic ecosystems. To test the hypothesis that this biotype was an opportunistic aquatic pathogen a series of studies on survival strategies in artificial and natural seawater microcosms as well as on routes of disease transmission were performed with serovar E strains. Results obtained suggested that serovar E strains were true aquatic bacteria since they survived in seawater microcosms for years maintaining its pathogenic potential (Marco-Noales *et al.*, 1999; Marco-Noales *et al.*, 2004) as well as used the water as main vehicle to infect new hosts (Amaro *et al.*, 1995; Marco-Noales *et al.*, 2001). However, the lack of positive water isolations contrasted to these laboratory data (Amaro *et al.*, 1999; Arias *et al.*, 1999; Høi *et al.*, 1998b). Our hypothesis was that the isolation methodology failed because it favored the growth of water bacterial competitors (among them biotype 1 strains) that could be deleterious for serovar E cells and inhibit their isolation. This hypothesis was based on the results obtained in survival experiments previously performed in water-microcosms co-inoculated with serovar E strains and selected competitors (Marco-Noales *et al.*, 2004). In those experiments, competition phenomena with other bacteria, which were detrimental for the recovery of serovar E strains from mixed populations were observed both in presence and absence of nutrients (Marco-Noales *et al.*, 2004).

The isolation of pathogenic *Vibrio spp.* is usually accomplished by cultural methods that start with enrichment in alkaline peptone water (APW; pH 8.6) with 1% NaCl to recover sub-lethal injured organisms, followed by plating onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Colwell, 1984). The enrichment step should improve the ratio of target to background microbiota before a selective plating step. Various enrichment broths have been tested for their capability to support the isolation of *V. vulnificus*, including APW with various salt concentrations,

marine broth, salt-polymyxin B broth, Horie's broth, Monsur's broth and glucose-salt-teepol broth (Arias *et al.*, 1998; Biosca *et al.*, 1997b; Hagen *et al.*, 1994; Sloan *et al.*, 1992). The procedure recommended in the Bacteriological Analytical Manual of the USFDA (Donovan and van Netten, 1995; US Food and Drug Administration, 1995), is an overnight enrichment in APW with 1% NaCl at 35-37°C followed by plating onto selective-differential media such as TCBS agar or CPC (cellobiose-polymyxin-colistin) agar or their derivatives, mCPC (modified CPC) agar, CC (colistin-cellobiose) agar and VVM (*Vibrio vulnificus* medium) (Cerda-Cuellar *et al.*, 2000; Høi *et al.*, 1998a; Massad and Oliver, 1987; Oliver *et al.*, 1992; Tamplin *et al.*, 1991).

Figure 5. Some of the selective media used in the *V. vulnificus* study.



This protocol was unsuccessfully used for the biotype 2 recovery by different laboratories, even from eel farm water during epizootics or outbreaks, when high numbers of viable cells are released to water and it was concluded that serovar E isolation was difficult because the pathogen did not survive or was present in very low numbers in the aquatic environment (Arias *et al.*, 1999; Cerda-Cuellar *et al.*, 2001; Høi *et al.*, 1998c; Macian *et al.*, 2000).

The main objective of the present study was to develop a new two-step protocol for the specific isolation of serovar E strains from asymptomatic eel carriers and water samples. The new protocol should favour growth of serovar E strains and inhibit that of putative competitors, especially those of biotype 1 which can be co-isolated from aquatic and fish samples and, according to Marco-Noales *et al* .(2004), induce a viable but nonculturable state in serovar E cells. For the first step, a selective and enrichment broth containing eel serum was designed. The selective properties of this broth rely on the bactericidal activity of fresh eel serum, to which serovar E cells are resistant (Amaro *et al.*, 1997). The efficacy of this new medium in supporting growth of serovar E cells and inhibit that of the putative competitors was evaluated and compared with of the APW by using selected *V. vulnificus* and *Vibrio spp* strains. Next, the optimal incubation conditions were established and the medium was tested with a large collection of putative competitors belonging to *V. vulnificus* (biotype 1 and biotype 3) and other aquatic bacterial species. For the second step, several selective and differential media were compared for their ability to recover serovar E strains, and the most efficient one was selected. The efficacy of the entire protocol was tested with natural environmental samples that included seawater, freshwater, eel tank water and healthy eel tissues. The results obtained clearly indicate the suitability of the new protocol for successfully isolating serovar E strains from natural mixed populations. Finally, the new serovar E isolates were characterized and compared with strains collected from clinical origins.

MATERIAL AND METHODS

Bacterial strains and growth conditions

A total of 37 *V. vulnificus* strains of different biotypes, sources and origins, as well as 23 strains of other species were used in this study (Tables 5 and 6). The strains were maintained both as lyophilized stocks at room temperature (25ºC) and as frozen stocks at -80ºC in Marine Broth (Difco) plus 20% (vol/vol) glycerol. Strains were routinely grown in Trypticase soy broth (TSB) or Trypticase soy agar (TSA) (Difco) supplemented with 0.5% (wt/vol) NaCl (TSB-1 and TSA-1, respectively) at 28ºC for 24 h. This growth conditions were used during all the PhD proyect unless other conditions were indicated.

Efficiency of the new selective enrichment broth

Saline eel-serum broth (SEB).

Pooled eel serum was obtained from eels as previously described (Esteve-Gassent *et al.*, 2003), and stored at –80ºC. Phosphate buffered saline (PBS) (KCl 0.02%, Na_2HPO_4 0.15%, KH_2PO_4 0.20%) supplemented with NaCl to a final concentration of 1% (PBS-1), 2% (PBS-2) or 3% (PBS-3), was adjusted to the pH of eel blood (pH = 8.3). SEB was prepared by diluting eel serum 1:5 in PBS-1 (SEB-1), PBS-2 (SEB-2) or PBS-3 (SEB-3) immediately before used.

Bacteria.

Sterile microcosms of artificial seawater (ASW) (Wolf and Oliver, 1992) were prepared in screw-cap glass tubes of 30 ml as previously described (Marco-Noales *et al.*, 1999), and were inoculated with bacterial suspensions from TSA-1 plates in ASW to a final concentration of 10^6-10^7 CFU per ml. All tubes were incubated in the dark in a static state at room temperature for at

least 1 month to simulate the starvation conditions typical of natural environments. Microcosms were sampled weekly for cultivable counts by the drop plate method (Hoben and Somasegaran, 1982) onto TSA-1 plates (Marco-Noales *et al.*, 1999).

Growth curves.

The effectiveness of SEB and APW in inhibiting the growth of competitors and enhancing the growth of serovar E strains was tested with pure cultures (Tables 5 and 6). Three independent experiments were performed with each strain and media tested. In each experiment, the enrichment broth was inoculated with starved cells (10^2-10^3 CFU per ml) and incubated with shaking at 28ºC for 10 h. Plate counts from single cultures were made on TSA-1 by drop plate (Hoben and Somasegaran, 1982) and samples were taken 0, 1, 2, 3, 4, 6, 8 and 10 h of incubation. The average and standard deviation of each bacterial counting per incubation time was calculated, and the significance of the differences between data was determined by variance analysis (ANOVA) performed with SPSS release 11.0 (SPSS, Inc). The most effective combination of broth and time of incubation was tested with all the strains listed in table 5 and 6 and with mixed cultures (serovar E plus one competitor). For this purpose, microcosms of these bacteria were prepared as described above and used to inoculate the enrichment broth. Plate counts from single and mixed cultures were made on TSA-1 and MSWYE-BTB (Marine seawater yeast extract with bromotimol blue plus 1% of mannitol) (Marco-Noales *et al.*, 2004) (on this medium, serovar E strains develop green colonies while colonies of the selected competitors are yellow), respectively, by drop plate methodology (Hoben and Somasegaran, 1982). Experiments were made in duplicate. The final growth rate (GR) was calculated for each strain as the log of final count/initial count and was coded as 0 ($GR \leq 1$), 1 ($1 < GR \leq 2$), 2 ($2 < GR \leq 3$), 3 ($3 \leq GR \leq 4$) or 4 ($4 \leq GR$).

Efficiency of selective media

Five selective media (TCBS agar (Difco), CC, CPC, mCPC and VVM agars) (see figure 5 for an example of *V. vulnificus* growth) were tested for efficiency in the recovery of *V. vulnificus* serovar E strains with respect to the general medium TSA-1. These selective media were prepared according to the original descriptions (Cerda-Cuellar *et al.*, 2000; Høi *et al.*, 1998a; Massad and Oliver, 1987; Tamplin *et al.*, 1991). Selected *V. vulnificus* strains (Table 5) were grown overnight in MSWYE at 28ºC and bacterial counting was made onto the selective and general media by the drop plate methodology. Plates were incubated for 24-48 h at the recommended temperatures; TSA-1 and TCBS agar at 28ºC, VVM at 37ºC (Cerda-Cuellar *et al.*, 2000), and CC, mCPC and CPC agar at 40ºC (Høi *et al.*, 1998a; Massad and Oliver, 1987; Tamplin *et al.*, 1991). To test the influence of the incubation temperature on serovar E recovery, CC, mCPC and CPC agar plates were also incubated at 37ºC. The efficiency at recovery was expressed as a recovery rate (RR) and was calculated as the percentage of CFU recovered on each of the selective media compared to the CFU obtained on the corresponding TSA-1 plate, and was expressed as the mean value ± standard deviation. A Mann-Whitney non-parametric analysis of variance was performed to evaluate the statistical significance of differences in the efficiency values (SPSS for Windows; release 11.0; SPSS, Inc). The most effective selective medium was tested for its effectiveness in inhibiting growth of the putative competitors. For this purpose, all the strains listed in Table 6 were grown in MSWYE at 28ºC for 18 h, and a loopful was streaked onto the selected agar medium. Plates were incubated at the recommended temperatures for 24-48 h and were examined for growth.

Table 5. Origin, biotype and serovar of the reference *V. vulnificus* strains used in this study.

Strain	Source	Group/ Serovar	Virulence [a] Eels/mice	GR in SEB
CECT 529[T]	Human blood	BT1	-/+	0
CECT 5164	Human blood	BT1	-/+	0
CECT 5167	Human blood	BT1	-/+	0
CECT 5168	Human blood	BT1	-/+	0
CECT 5169	Human blood	BT1	-/+	0
ATCC 33816	Human blood	BT1	-/+	0
94385	Leg wound	BT1	-/ND	0
V4	Human blood	BT1	-/ND	0
CECT 5165	Sea water	BT1	-/ND	0
CECT 4608	Eel farm water	BT1	-/-	0
JE	Oyster	BT1	-/ND	0
VV425	Oyster	BT1	-/ND	0
CG100	Oyster	BT1	-/+	0
CG106	Oyster	BT1	-/ND	0
CG110	Seawater	BT1	-/ND	0
A2	Eel farm water	BT1	-/-	0
CECT 4601	Diseased eel	BT2-SerE	+/+	4
CECT 4604	Diseased eel	BT2-SerE	+/+	4
CECT 4605	Diseased eel	BT2-SerE	+/+	4
CECT 4917	Diseased eel	BT2-SerE	+/+	4
CECT 4864	Diseased eel	BT2-SerE	+/+	4
CECT 4865	Diseased shrimp	BT2-SerE	+/ND	4
Ö122	Diseased eel	BT2-SerE	+/ND	4
CECT 5198	Diseased eel	BT2-NonSerE A	+/ND	4
CECT 5343	Diseased eel	BT2-NonSerE O3	+/ND	3
CECT 5768	Diseased eel	BT2-NonSerE	+/ND	1
95-8-6	Diseased eel	BT2-NonSerE	+/ND	1
95-8-7	Diseased eel	BT2-NonSerE O3	+/ND	1
95-8-161	Diseased eel	BT2-NonSerE O3:O4	+/ND	2
95-8-162	Diseased eel	BT2-NonSerE O3:O4	+/ND	4
Bt3-97	human	BT3	-/ND	1
Bt3-vv12	human	BT3	-/ND	0
Bt3-11028	human	BT3	-/ND	1

Strain	Source	Group/ Serovar	Virulence [a] Eels/mice	GR in SEB
Bt3-58	human	BT3	-/ND	ND
Bt3-1033	human	BT3	-/ND	2
Bt3-vv32	human	BT3	-/ND	1
Bt3-162	human	BT3	-/ND	0

[a] Data on virulence from (Biosca, 1994; Fouz and Amaro, 2003)

* Strain selected for growth curves of single and mixed populations in selective enrichment broth

Table 6. Origin and virulence of the reference strains other than *V. vulnificus* used in this study

Species	Strain	Origin	Source	Virulence for [a] eels	GR in SEB	Growth in VVM
V. harveyi	CECT 604	Seawater	Spain	-	0	-
	CECT 605	Marine plankton	Spain	ND	0	-
	CECT 606	Marine plankton	Spain	-	0	-
	CECT 607	Seawater	Spain	ND	4	-
	CECT 608	Seawater	Spain	-	0	-
V. alginolyticus	CECT 521^T	Diseased horse mackerel	Japan	-	0	-
	CECT 600	Seawater	Spain	ND	0	-
	CECT 601	Seawater	Spain	ND	0	-
	CECT 603	Marine plankton	Spain	ND	0	-
	CECT 610	Marine plankton	Spain	ND	0	-
	RA3	Diseased amberjack	Spain	ND	0	-
V. mediterranei	CECT 615	Marine plankton	Spain	-	0	+(G)[b]
	CECT 621^T	Marine sediment	Spain	-	0	+(G)[b]
V. splendidus	CECT 528	Marine fish	Spain	-	0	-

Species	Strain	Origin	Source	Virulence for [a] eels	GR in SEB	Growth in VVM
	CECT 4204	Seawater	USA	-	0	-
V. parahaemolyticus	CECT 611	Seawater	Spain	-	0	-
	CECT 612	Seawater	Spain	ND	0	-
	CECT 613	Marine plankton	Spain	ND	0	-
V. spp	PD-4*	Eel tank water	Spain	ND	0	-
Edwadsiella tarda	CECT 886	Eel tank water	USA	+	0	-
Pseudomonas anguilliseptica	CECT 899^T	Diseased eastern fish	Japan	+	0	-
Aeromonas hydrophila	E37	Diseased eel	Spain	+	1	-
Aeromonas jandaei	S345	Healthy eel	Spain	ND	4	-

[a]: Data of virulence from Biosca *et al* (9) and Esteve *et al*(20)

[b]: G, green colonies.

* Strain selected for growth curves of single and mixed populations in selective enrichment broth

Field samplings

A total of 8 samples of water from a Spanish coastal site (Delta del Ebro), a freshwater lake (Albufera Lake) and two eel-farms, as well as 9 samples of tissues taken from wild and cultured eels were tested (see results: Table 7). One of the farms had experienced recurrent outbreaks of vibriosis two years before (Table 7). In the case of water samples, 250 ml volumes were taken in sterile flasks and filtered through sterilized 0.22 μm-pore size

membrane filters (Millipore). In eels, samples of mucus, spleen, gills and liver from four eels per sampling were aseptically taken, pooled and homogenized in PBS-1. In the case of glass-eels, the whole animals were homogenized in PBS-1. Filters and tissue homogenates were incubated by shaking in flasks containing 250 ml APW or tubes containing 25 ml of the selected SEB at 28ºC for 18 and 8 h (see results section), respectively. Tenfold dilution in PBS-1 of all enrichments were performed, and aliquots of 100 µl were streaked onto the selective media selected in prior experiments. An average of 30 suspected colonies from each sampling were purified on TSA-1 for further studies.

Identification and characterization of the environmental isolates.

Identification of the species.

For identification to the species level, colony hybridization with a *V. vulnificus* specific alkaline phosphatase-labeled (VVAP) DNA probe directed against a cytolysin-hemolysin gene was used (Wright *et al.*, 1993).

Serological identification and biochemical characterization.

Confirmed *V. vulnificus* isolates were serologically identified by slide agglutination with previously obtained rabbit polyclonal antibodies anti-serovar E, anti-serovar A, and with two other sera obtained against two strains that were previously serotyped with monoclonal antibodies as O3 and O3/O4 (Amaro *et al.*, 1992b; Fouz and Amaro, 2003). A total of 9 randomly selected serovar E strains were seeded in API 20E and API 20NE strips (Biomerieux, Spain), and the API profiles were compared with the API DataBase (APILAB Software, version 3.3.3, Apilab Plus; Biomerieux) Additionally, the following biochemical tests were performed: Simmons citrate (CIT), Thornley's arginine dihydrolase (ADH) and decarboxylation of lysine and ornithine in Mueller broth (decarboxylase medium base, Difco) (LDC, ODC) (Biosca *et al.*, 1993a).

Serum resistance.

The sensitivity to eel and human serum of the selected *V. vulnificus* serovar E strains was evaluated with TSA-1-grown bacteria in microtiter plates (Amaro *et al.*, 1999). In each well, a volume of 50 µl of eel serum or iron-overloaded human serum [supplemented with 10 µM Desferoxamine mesylate (Desferal, Sigma)] (Amaro *et al.*, 1994) was mixed with 50 µl of a suspension of bacteria (10^3-10^4 CFU / ml) in PBS-1. Assays were made in triplicate, taking samples at 0, 1, 2, 3 and 4 hours of incubation at room temperature. Viable counts were determined by drop plating on TSA-1.

Virulence assays.

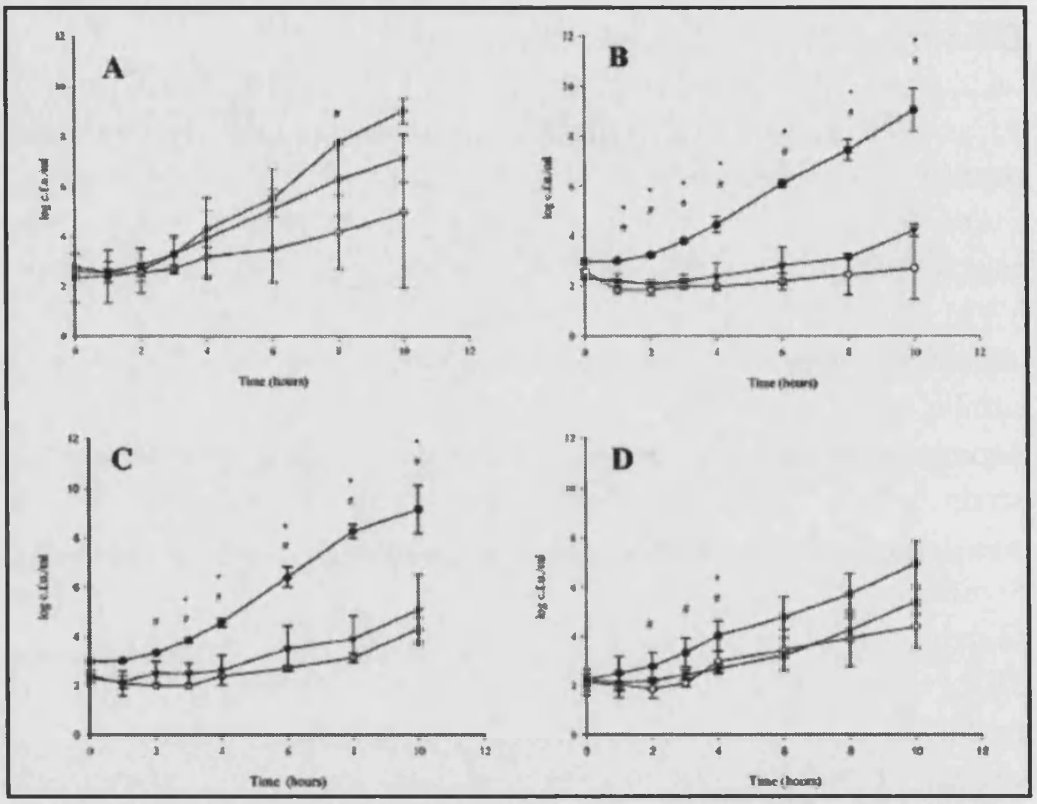
The potential virulence of the *V. vulnificus* serovar E isolates for humans and eels was tested by using BALBc mice of 20 g of average weight (5-6 weeks old) and juvenile European eels of 10 g of average weight (elvers), respectively. Groups of six animals were used in each experiment that lasted 1 week. Mice were pre-treated two hours before with Desferal (250 µg/g of body weight) as the virulence of *V. vulnificus* is markedly increased when serum contains this iron chelator (a hydroxymate-type siderophore that promotes growth in serum; "(iron-overloaded mice") (Amaro *et al.*, 1994). Eels were maintained in aerated tanks containing 6 litters of saline water (1% NaCl) at 25ºC (Amaro *et al.*, 1995). The *V. vulnificus* serovar E environmental isolates were grown in TSA-1 at 28ºC for 24 h, and bacterial suspensions in PBS-1 were prepared. Approximately, 10^4 CFU/fish and 10^3 CFU/mouse were injected into elvers (0.1 ml per fish) or mice (0.2 ml per mouse). Appropriate controls for each experiment were also included (mice and elvers inoculated with PBS-1) (Amaro *et al.*, 1995; Amaro *et al.*, 1994). Mice and elver mortalities were recorded daily and only considered if the bacteria were isolated in pure culture from internal organs.

RESULTS

Efficiency of the selective enrichment broths

The growth curves in SEB of starved cells of the selected serovar E, *V. vulnificus* biotype 1 and Vibrio spp. strains (see Table 5 and 6) are shown in Figure 6. The strain of *Vibrio spp.* was selected because it was co-isolated with *V. vulnificus* biotype 1 on VVM from water of an eel-tank in a routine control study. *V. vulnificus* biotype 1, serovar E and *Vibrio spp.* strains showed similar growth patterns in APW without significant differences between the curves (Figure 6). In contrast, the growth of serovar E strain in SEB-1 and SEB-2 was significantly higher than that of the non-target bacteria throughout the incubation period (Figure 6). Significant differences were also observed in SEB-3, but only in the fourth hour of incubation (Figure 6). The generation times (g) in SEB-1 and SEB-2 of the non-target bacteria were very similar (around 1 h in SEB-1 and 0.7 h in SEB-2) and longer than those of the target bacteria (around 0.4 h in both media) (Figure 6). In contrast, growth of both target and non-target were very similar in SEB-3 (around 0.75 h) (Figure 6). The greatest differences in the final GR between the target and non-target bacteria were found in SEB-1 after 8 h of incubation ($3x10^7$-$2.5x10^9$ versus $1x10^3$-$2x10^4$). Thus, SEB-1 was co-inoculated with one serovar E strain plus one competitor (Tables 5 and 6) in a proportion 1:1. After 8 hours of incubation, only green colonies could be count in the experiments regardless of the strain co-inoculated with the serovar E strain. Finally, SEB-1 was tested with biotype 2 strains of other serovars (Table 5), of biotypes 1 and 3 (Table 5) and of selected competitor species (Table 6). After 8 h of incubation, only four strains, two of biotype 2, one of *V. harveyi*, and the other of *A. jandaei*, achieved the same GR as the serovar E isolates. Five biotype 2 strains and four biotype 3 isolates grew with GR values between 1 and 3, and the rest of strains did not grow (Tables 5 and 6).

Figure 6. Results of the selective enrichment broths.



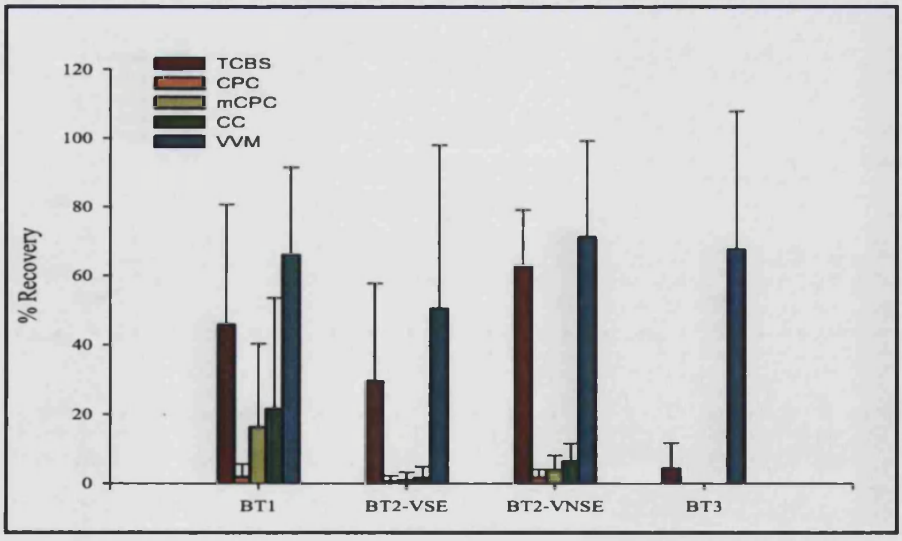
Growth curves in APW (A), SEB-1 (B), SEB-2 (C) and SEB-3 (D) of *V. vulnificus* CECT 4604 (•), CECT 529^T (○) and *Vibrio spp.* PD-4 (▼).
Each point represents the average ± SD of values from at least three different experiments. Significant statistical differences (α = 0.05) between CECT 4604 and CECT 529^T (#) and between CECT 4604 and *Vibrio* spp. PD-4 (*) are indicated.

Efficiency of the selective media

The strains of *V. vulnificus* were classified into four groups: biotype 1, biotype 2 serovar E, biotype 2 non-serovar E and biotype 3 (Table 5). The recovery rates (RR) obtained for each group and for each selective medium are shown in Figure 7. Although high standard deviations within each group were observed, statistical analysis revealed significant differences between groups and media. No significant differences in colony counts were registered regarding incubation time (24 h or 48 h) or, in the case of CPC, mCPC and CC agars, the incubation temperature (40 or 37ºC) ($P \leq 0.05$). In all cases, the lowest RRs were seen on CPC agar (from

0.02 to 1.75%), and the highest ones on VVM (from 50.42% to 71.23 %) (Figure 7). *V. vulnificus* serovar E and biotype 3 strains did not grow well on mCPC agar and CC agar, which does not contain polymyxin B (Høi *et al.*, 1998a), and biotype 3 strains did not grow on TCBS agar either. In addition, statistical differences between groups regarding the RR on several media were detected. Thus, i) biotype 1 group showed RR significantly higher on TCBS, CC and mCPC agars than biotype 3 group, and on CC and mCPC agars than serovar E group; ii) biotype 2 non-serovar E group gave RR significantly higher on TCBS and CC agar than serovar E and biotype 3 groups; and iii) Serovar E yielded RR significantly higher on TCBS agar than the biotype 3 group. Since the highest RR of serovar E strains was achieved with VVM, this medium was selected for further experiments. On this medium, the three biotypes of *V. vulnificus* developed bright colonies of about 2 mm, biotypes 1 and 2 being yellow with a yellow diffusion halo, and biotype 3 being green without a halo (see Figure 5). The effectiveness of VVM in inhibiting growth of the putative competitors was tested with the strains listed in Table 2. Only *V. mediterraneii* strains were able to grow on VVM, giving green colonies of similar morphology to those of biotype 3.

Figure 7. Recovery rates (%) of the different groups of *V. vulnificus* on the selective media.



Field samplings

V. vulnificus was isolated from 7 out of 8 water samples and from 3 out of 9 tissue samples with both isolation procedures (Table 7). Regarding water samples, *V. vulnificus* was isolated from seawater (2.5% salinity) and tank water from fresh- and brackish-water eel-farms but not from lake water (Table 7). In the case of eel tissue samples, *V. vulnificus* was recovered from both cultured and wild eels (Table 3). After enrichment in APW, more than 95% of the presumptive yellow colonies on VVM were identified as belonging to *V. vulnificus* by colonial hybridization with the VVAP probe, but none of the isolates agglutinated with serovar E specific antisera. After enrichment in SEB-1, 100% of the presumptive colonies were identified as *V. vulnificus*. Serovar E was recovered from 3 out of 7 *V. vulnificus*-positive (VV+) water samples (seawater and eel-farm water) and 1 out of 3 VV+ tissue samples (cultured eels) (Table 7). The percentage of confirmed serovar E colonies ranged from 10-50% in the case of water samples, to 100% in the case of eel samples (Table 3). Around 20 randomly selected non-serovar E isolates from water and eel samples were tested with serovar A, serovar O3 and serovar O3/O4 specific antisera. Two isolates (10%) from tank-water agglutinated with anti-serovar A sera and the rest (50%) did not agglutinate with these sera or were autoagglutinating (40%).

Table 7. Isolation of *V. vulnificus* serovar E from water and fish samples by the APW+VVM and SEB-1+VVM procedures

Type of sample[a] (n)	Eel body weight (g)	Physico-chemical parameters of water[b]		No. positive samplings for V. vulnificus/V. vulnificus serovar E[c]	
		T[a] (°C)	Salinity (%)	APW+VVM	SEB+VVM
Water from (8)					
Ebro delta (1)		25	2.5	1/0	1/1 (50%)
Albufera Lake (1)		25	0.5	0/0	0/0
Freshwater eel-farm (3)		25-28	0.1	3/0	3/0
Brackish water eel-farm (3)		25-28	1.5-1.7	3/0	3/2 (10%, 50%)
Tissues from (9)					
Wild glass-eels (3)	0.1	-	-	0/0	0/0
Wild eels (2)	275	-	-	1/0	1/0
Cultured elvers (4)	10	25-28	0.1	2/0	2/1 (100%)

[a]All samplings of water and eels from eel-farms were performed in the absence of epizootics or outbreaks. The cultured elvers came from the freshwater eel-farm, which had experienced epizootics of vibriosis two years before the sampling. Wild glass-eels came from the Mediterranean Sea and adult eels from Albufera Lake, and were bought alive in a public market in Valencia (Spain).

[b]The temperature and salinity of tank-water from which cultured eels were cultured is also indicated.

[c]The percentage of serovar E *versus V. vulnificus* colonies growing onto VVM is indicated in parenthesis.

Characterization of the environmental serovar E strains.

Nine randomly selected serovar E isolates were further characterized. The origin of these strains and the results of the API 20E and NE systems, as well as some additional conventional taxonomic tests are summarized in Table 8. Eight of these strains

gave the same API 20E profile as the control clinical Serovar E strain, while one differed only in citrate utilization. According to the API data base, the major profile corresponds to *V. vulnificus* with a probability of 54.4%, while the minor profile had a probability of 10.4% (Table 8). Due to previously reported discrepancies between conventional and commercial assays (Biosca *et al.*, 1993a) several tests were re-examined. More than 80% of the isolates were positive for citrate by the conventional test, and more than 60% for ODC, a phenotypic trait that had a negative result in the API 20E system. Regarding the API 20NE system, 7 isolates gave the same profile as the control strain which, according to the API data base, did not correspond to *V. vulnificus* (Table 4). The other two strains showed slight differences, and only one was identified as *V. vulnificus* with a probability of 75% (Table 8).

All serovar E isolates could survive and grow in non-diluted fresh eel and iron-overloaded human sera, giving bacterial yields similar or even higher to those of the control strain (Figure 8). In addition, all strains were virulent for eels and iron-overloaded mice, giving a percent mortality higher than 75% after the injection of 10^4 CFU/fish or 10^3 CFU/mouse. Eel and mice mortalities occurred before 48 h, and bacteria were recovered as pure cultures from internal organs. The infected eels showed redness on the body, particularly on the head and tail. Internally, the liver and kidney appeared hemorrhagic as did the muscle wall on occasion. No external pathological signs were observed in moribund mice except for occasional small ulcers on the tail.

Table 8 Biochemical characteristics of the selected *V. vulnificus* serovar E strains isolated from water and asymptomatic carriers.

Strain	Source	API20E profile	API20NE profile	Conventional tests:			
				CIT	ADH	LDC	ODC
PD-8	Eel tank water	5006005[b]	5473745[d]	-	-	+	-
AnS1	Gills	5006005[b]	5472645[e]	+	-	+	+
C1	Liver	5006005[b]	5472745[f]	+	-	+	-
Riu-2	Seawater	5006005[b]	5472745[f]	+	-	+	-
PD-2-47	Tank water	5006005[b]	5472745[f]	+	-	+	+
PD-2-50	Tank water	5006005[b]	5472745[f]	+	-	+	+
PD-2-51	Tank water	5006005[b]	5472745[f]	+	-	+	+
PD-2-55	Tank water	5006005[b]	5472745[f]	+	-	+	-
PD-2-56	Tank water	5206005[c]	5472745[f]	+	-	+	+
CECT 4604[a]	Diseased eel	5006005[c]	5472745[f]	+	-	+	-

[a]. *V. vulnificus* servovar E control strain.

[b]. Identification according to the API data base: *V. vulnificus* (54.4%), *Burkholderia cepacia* (45.3%).

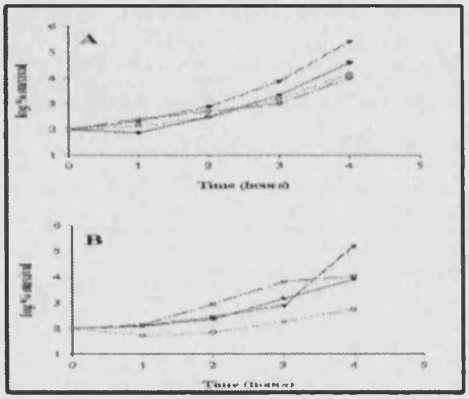
[c]. Identification according to the API data base: *Bl. cepacia* (89.3%), *V. vulnificus* (10.4%).

[d]. Identification according to the API data base*: A. hydrophila/caviae* (94.5%);

[e]. Identification according to the API data base: *V. vulnificus* (75%), *V. cholerae* (17.4%), *V. alginolyticus* (4.6%)

[f]. Identification according to the API data base: *V. cholerae* (45%), *A. hydrophila/caviae* (41.7%)

Figure 8. Serum resistance of new isolates.



Growth curves in eel (A) and iron-overloaded human (B) serum of the environmental serovar E strains PD-8 (•), AnS1 (○) and C1 (▼) with respect to the control strain CECT 4604 (▽). Each point represents the average of three different experiments

ionic potential and make the more sensitive strains (serovar E and biotype 3) more resistant to polymyxins. VVM was tested with a wide collection of strains belonging to putative competitor species and only *V. mediterranei* grew, developing green colonies. Nevertheless, since this species was inhibited by SEB-1, its presence in a sample should not cause problems.

In the second part of this study the protocol was validated in the field using APW + VVM as control. Firstly, *V. vulnificus* was isolated with both protocols from water and healthy tissue samples. However, the percentage of positive isolations was considerably higher to that previously reported for other sites of the Mediterranean Sea of higher salinity (Arias *et al.*, 1999; Macian *et al.*, 2000), and similar to that found in other habitats of similar salinity (Cerda-Cuellar *et al.*, 2001; Høi *et al.*, 1998a). Serovar E strains were only recovered after SEB-1 enrichment, which confirmed that the competition phenomena present in APW could be abolished by using this selective enrichment broth. In fact, 43% and 33.33% of the samplings positive for *V. vulnificus* from water and tissues, respectively, were also positive for serovar E recovery, which constituted between 10 and 100% of the *V. vulnificus* colonies, depending on the sample. In addition, after SEB enrichment, serovar A strains, together with other non-typeable strains, were also isolated. Interestingly, serovar E was not detected in wild eels. Although more samplings are needed, this result supports the hypothesis that wild European eels are not the natural host for this serovar, which underlines the highly virulent potential of this pathogen for non-immunized eels (Amaro *et al.*, 1997). All the selected environmental isolates were clearly identified as serovar E strains since they agglutinated with the specific antiserum. These isolates were virulent for eels and iron-overloaded mice and resisted the bactericidal action of eel and iron-overloaded human sera. The biochemical profiles of the serovar E isolates were quite homogeneous and similar to the clinical control serovar E strain included in this study. In contrast to the report of O'Hara *et al.* (2003), we found that all serovar E strains were negative for the indole test on the API 20E, which is in accordance

with previous studies (Amaro *et al.*, 1992b; Biosca *et al.*, 1991; Biosca *et al.*, 1997a; Biosca *et al.*, 1993a; Biosca *et al.*, 1996b; Muroga *et al.*, 1976; Tison *et al.*, 1982). Some discrepancies in the results of CIT and ODC tests, already described (Biosca *et al.*, 1993a), were detected since most of the environmental isolates were positive for both characters in conventional tests but not in commercial ones. In addition, none of the isolates was correctly identified by the API 20 E and NE system, which shows the limited value of these systems for the identification of *V. vulnificus* unless these profiles are included in the API data base.

In conclusion, a new two-step protocol for serovar E specific isolation from environmental samples has been developed and validated in the present work. The key element of this protocol was the enrichment broth used in the first step, which yielded differences in bacterial counts between serovar E and competitors high enough to allow the isolation of this serovar from natural, mixed populations after only 8 h of incubation. For the second step, the selective and differential agar *Vibrio vulnificus* medium (VVM) was selected because it gave the highest plating efficiency not only for the serovar E group but also for the rest of *V. vulnificus* groups, including biotype 3. The entire protocol was validated in the field since serovar E strains were, for the first time, isolated from sea water and eel-farms in the absence of epizootics or outbreaks. Finally, the overall results demonstrate that serovar E strains are present in the aquatic environment and that only with adequate procedures of isolation their true epidemiological relevance will be revealed.

DISCUSSION

In the present study, a two-step protocol for the isolation of serovar E strains from asymptomatic carriers and environmental samples was developed and validated with field samples. Selected target and non-target bacteria grew equally well in the enrichment broth recommended by the US FDA, which confirmed that APW is not adequate for the selection of serovar E strains from a pool of *V. vulnificus* strains. Fresh eel serum diluted in alkaline PBS (SEB) was chosen because eel virulence in *V. vulnificus* is related to resistance to serum complement (Amaro *et al.*, 1997), as well as the growth rate in eel serum (Amaro *et al.*, 1997; Esteve-Gassent *et al.*, 2004a). Until now, animal sera had only been used after heat-inactivation as a nutrient supplement for the growth of some fastidious pathogenic bacteria (Jiang and Doyle, 2000; Watanabe, 1994). The present approach takes advantage of the ability of these bacteria to cause infection (grow in eel serum) as a means of selecting them from a pool of competitors. The results obtained with pure and mixed cultures confirmed that SEB acted as a culture broth for serovar E strains, while it was bacteriostatic or bactericidal for the competitors, including the biotypes 1 and 3 strains of this species. The optimum selective conditions were obtained by incubating bacteria in SEB-1 for 8 h at 28 ºC. In these conditions serovar E cells could be selected even from a pool of biotype 2 cells on the basis of the higher growth rates of the serovar E strains in eel serum.

With regard to the valuation of the efficacy of the selective media, the results clearly demonstrated that CPC agar should not be used for serovar E recovery (RR<0.5). The inability of serovar E strains to grow in CPC agar had previously been reported by Macian *et al.* (2000) and suggested by Høi *et al.* (1998a). Furthermore, this inability was generalized for all the *V. vulnificus* groups, including the biotype 1 group (RR<2), which contrasts with the data reported by other authors (Cerda-Cuellar *et al.*, 2000; Høi and Dalsgaard, 2000; Høi *et al.*, 1998a). This discrepancy could be

due to differences in the physiological state of cells since they were previously grown in a low nutrient medium (MSWYE) instead of a high nutrient one (TSA and others) as usual. mCPC and CC agars, which contain less antibiotics, are recommended instead of CPC agar by many investigators, including the FDA which recommends mCPC agar. Nevertheless, as shown in this study, these CPC derivatives were also inadequate for the recovery of serovar E (RR below 1.5%), non-serovar E (RR below 6%) and biotype 3 strains (RR below 0.02%), although the RR of biotype 1 strains increased significantly (around 20%). These results could explain by themselves why no biotype 2 and 3 strains have been isolated from the environment. The efficacy in the recovery of all groups except one (biotype 3) increased significantly when TCBS agar was employed. This medium is widely used for the isolation of the genus *Vibrio* (Arias *et al.*, 1999; Hervio-Heath *et al.*, 2002; Montanari *et al.*, 1999; Pfeffer *et al.*, 2003; West *et al.*, 1982) and is the second medium recommended by the FDA for the isolation of *V. vulnificus*. However, its efficacy has been questioned by several authors (Cerda-Cuellar *et al.*, 2000; Høi *et al.*, 1998a; West *et al.*, 1982), primarily due to the reproducibility of the results. In fact, we obtained RR values much higher than those reported by other laboratories (Høi *et al.*, 1998a). In addition, the RR on TCBS of serovar E strains was significantly lower than those of other biotype 2 strains. This result could be related to the etiological characteristics of the diseases since non-serovar E strains colonize the intestines and mostly produce intestinal hemorrhages and feces with blood (Fouz and Amaro, 2003). Oddly enough, serovar E strains were more similar to biotype 3 strains, and non-serovar E isolates to biotype 1 isolates, regardless of the strains' origin. This apparent subdivision of the eel pathogenic group supports the polyphyletic origin proposed by Gutacker *et al.* for the biotype 2 strains of the species (Gutacker *et al.*, 2003). Fortunately, VVM significantly increased the efficacy in the recovery of all groups, without differences between eel pathogenic subgroups. This medium contains electrolytes ($MgCl_2.6\ H_2O$ and KCl) that stimulate growth of pathogenic vibrios (Donovan and van Netten, 1995). The binding of these cations to the bacterial membrane could alter its

4

IDENTIFICATION OF DNA SEQUENCES SPECIFIC TO BIOTYPE 2 *V. vulnificus* STRAINS BY SUPPRESSION SUBTRACTIVE HYBRIDIZATION

INTRODUCTION

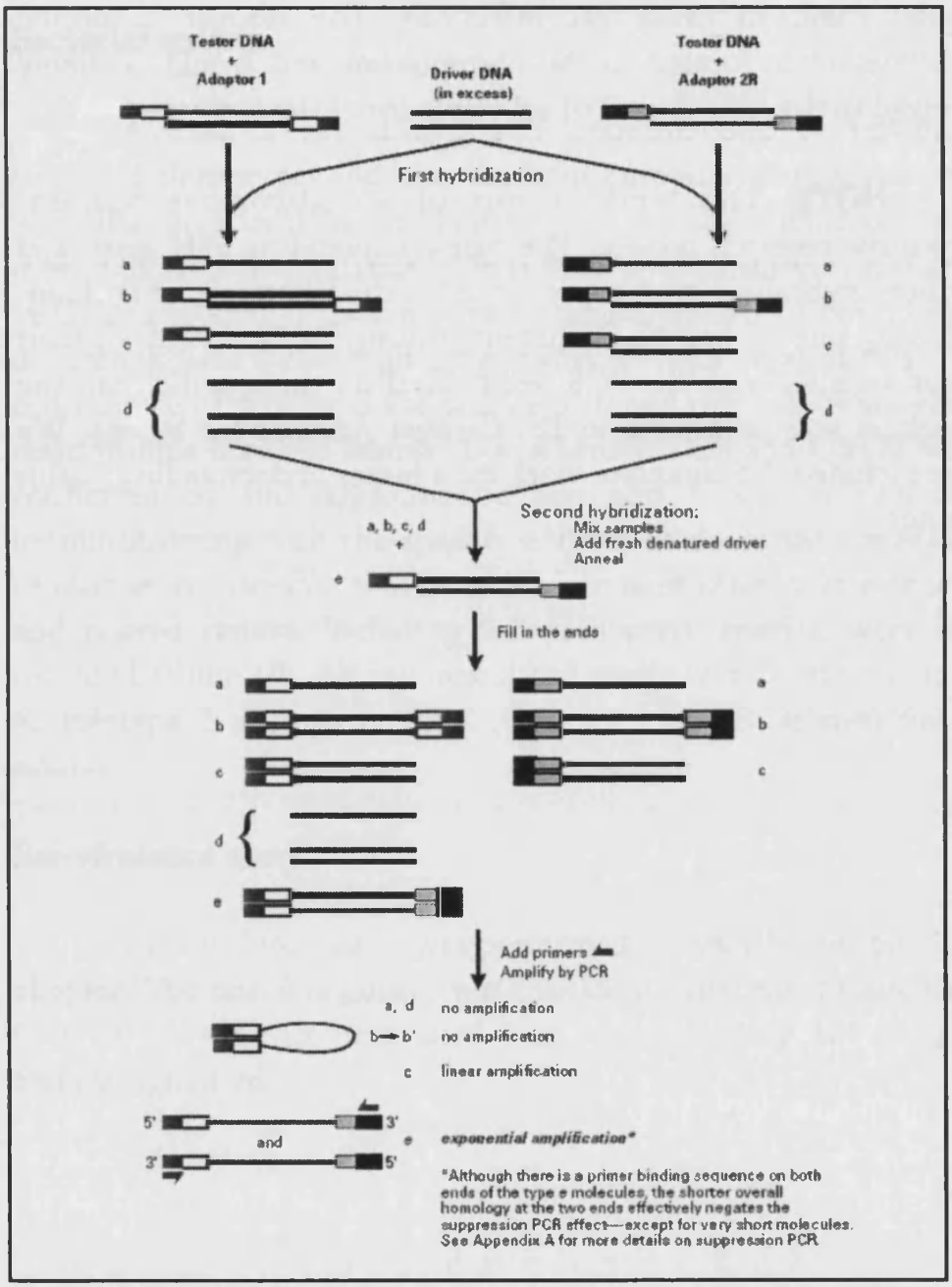
What makes some strains of a pathogenic bacterial species more virulent compared with close relatives? Why strains of the same species can infect different hosts? These differences are associated with genomic regions present only in the virulent strains. Various virulence-associated genes have been found on motile genetic elements or in variable regions of the chromosome; such as transposons, integrons, plasmids, or phages (Hacker *et al.*, 2003a; Hacker *et al.*, 2003b). In the last decades, with the introduction of large-scale genome sequencing projects and the development of good techniques for comparative genomics, we have experience an increasing knowledge of the organization of the bacterial genomes. These studies have revealed that sometimes the virulence determinants are clustered in a large region of the chromosome that is not present in the nonpathogenic strains; these regions are denominated pathogenicity islands (PAIs) (Hacker *et al.*, 2003a).

When we reviewed, in the General Introduction, the virulence factors described in *V. vulnificus*, we commented that they are present in all the strains although a great diversity in pathogenicity is observed in this species; for example the strains of the biotype 2 are the only ones in the species defined as virulent for fish (Tison *et al.*, 1982) and a part of them, serovar E, being also virulent for human as the other two biotypes (Amaro and Biosca, 1996). The virulence mechanism of the biotype 2 strains in eel vibriosis remains unclear, although a few virulence factors have been proposed. Since now, two surface factors of the serovar E strains have been related to eel virulence: the O-polysaccharide of LPS that is involved in resistance to eel serum complement (Amaro *et al.*, 1997), and the capsule that is essential for infecting the eels through water (Amaro *et al.*, 1995). Our hypothesis is that biotype 2 strains share exclusive genetic information than enable them to develop vibriosis in eels. A genome comparison between biotypes would help us to identify this DNA sequences specific for biotype 2

strains and discover the virulence factors responsible for eel vibriosis.

Suppression subtractive hybridization (SSH), a technique originally developed to study gene expression in eukaryotes (Diatchenko *et al.*, 1996), has been successfully used to identify strain- or species-specific DNA sequences in a variety of bacteria (Winstanley, 2002). SSH has been employed to identify the genomic islands implicated in virulence in *Helicobacter pylori* (Akopyants *et al.*, 1998), *Klebsiella pneumoniae* (Lai *et al.*, 2000), *Salmonella spp.*(Bogush *et al.*, 1999), *Burkholderia mallei* (22), *A. hydrophila* (Zhang *et al.*, 2000), *Serratia marcescens* (Zhang *et al.*, 2005), or *Yersinia spp.* (Wang *et al.*, 2006). In this technique (Figure 9), regions of DNA presents in a strain, designed as **tester**, but absent in another strain, designated as **driver**, are detected; briefly, tester DNA is digested with a restriction endonuclease (such as RsaI) and separated into two portions, each of which is subjected to a ligation reaction to attach a different adaptor sequence to the 5' ends. The portions are then separately hybridised to driver strain DNA (in excess). Any sequences that hybridise with the driver DNA should be 'mopped up', leaving only tester-specific single-stranded sequences. When the two portions are eventually mixed and hybridised together, allowing homologous single-stranded DNAs to hybridise, only those sequences unique to the tester strain will have different adaptors present on each strand. These sequences are detected by PCR amplification with primers designed to bind to adaptor sequences. The key to the success of this strategy is that sequences containing the same adaptor sequence at both ends are unable to amplify because of a suppression effect whereby a secondary structure forms to prevent primer annealing (Clontech PCR-SelectTM Bacterial Genome Subtraction Kit User Manual: PT3170-1). Consequently, only those sequences now carrying both adaptors (one at each end) will amplify. These PCR products are cloned into a suitable vector to produce a subtracted DNA library.

Figure 9. Schematic diagram of the SSH.



Adapted from Clontech (PCR-SelectTM Bacterial Genome Subtraction Kit).

In this study, we identified the *V. vulnificus* biotype 2-specific DNA sequences by SSH, and found that some of these DNA sequences are common to all the biotype 2 strains while some others are specific to the serovar E strains. The sequences common

to all biotype 2-specific sequences were plasmid-borne, suggesting that the biotype 2 strains may share a virulence plasmid that enables them to cause eel infections. The serovar E-specific sequences were located in the chromosome and would be likely involved in the high degree of eel virulence of this serovar.

NOTE: This work is part of a collaborative Spanish-Taiwanese research project. The part comprending SSH assay and southern hybridization was performed in the laboratory of Dr Lien-I Hor by one of her Ph.D student, Chung-Te Lee. And virulent assays together with the PCR test to confirm the specificity of the sequences was performed in Dr. Carmen Amaro's lab by me. We have included the complete work for a better understanding of this chapter.

MATERIALS AND METHODS

Bacterial strains.

A total of 111 clinical and environmental *V. vulnificus* strains of all biotypes and serovars from Europe, America, Asia and Australia were used in this study (Table 9). The identification of these strains was confirmed by colony hybridization with a *V. vulnificus*-specific, alkaline phosphatase-labeled DNA probe directed against a cytolysin gene, *vvhA* (Wright *et al.*, 1993). The equivalence of serovar E and serovar O4, and the lack of antigenic relationships between serovar E and serovars O3 and O3:O4 were confirmed by slide-agglutination test plus LPS extraction and immunoblotting with the specific antisera as described previously (Biosca *et al.*, 1996b). A total of 37 strains of other vibrio species and related genera, including fish-pathogenic species, were also included (Table 10). All the inoculated media were incubated at 25 ºC (biotype 2 isolates) or 37ºC (biotypes 1 and 3 isolates) for 24 hours.

Eel-virulence assay.

Eel virulence assay was performed as described in previous chapter. The tested organism was considered virulent if more than 50% of the animals inoculated with approximately 10^6 cfu/g of body weight died.

Table 9. *V. vulnificus* used in this chapter.

Strain	Source	Country, yr	Serovar [a]	Virulence for eels
CECT 897	Diseased eel	Japan, 1979	E	+
CECT 898	Diseased eel	Japan, 1979	E	+
CECT 4601	Diseased eel	Spain, 1989	E	+
CECT 4602*	Diseased eel	Spain, 1990	E	+
CECT 4603	Diseased eel	Spain, 1990	E	+
CECT 4604	Diseased eel	Spain, 1990	E	+
CECT 4605	Diseased eel	Spain, 1990	E	+
CECT 4607	Diseased eel	Spain, 1992	E	+
CECT 4862	Diseased eel	Japan, 1979	E	+
CECT 4863	Leg wound	USA	E	+
CECT 4864	Diseased eel	Spain, 1994	E	+
CECT 4865	Diseased shrimp	Taiwan	E	+
CECT 4866	Human blood	Australia	E	+
CECT 4868	Diseased eel	Norway, 1990	E	+
CECT 4870	Diseased eel	Sweden, 1991	E	+
CECT 4917	Diseased eel	Spain, 1997	E	+[b]
CECT 4998	Diseased eel	Spain, 1997	E	+[b]
CECT 4999	Diseased eel	Spain, 1999	E	+[b]
CECT 5139	Diseased eel	Spain, 1998	E	+[b]
Ö122	Diseased eel	Sweden, 1995	E	+[b]
CECT 5763	Eel tank water	Spain, 2002	E	+[b]
CECT 5762	Healthy eel	Spain, 2002	E	+[b]
C1	Healthy eel	Spain, 2003	E	+[b]
PD-2-47	Eel tank water	Spain, 2003	E	+[b]
PD-2-50	Eel tank water	Spain, 2003	E	+[b]
PD-2-51	Eel tank water	Spain, 2003	E	+[b]
PD-2-55	Eel tank water	Spain, 2003	E	+[b]
PD-2-56	Eel tank water	Spain, 2003	E	+[b]
Riu 2	Seawater	Spain, 2003	E	+[b]
90-2-11	Diseased eel	Denmark, 1990	E	+[b]
94-8-112	Wound infection	Denmark, 1994	E	+[b]
94-9-123	Seawater	Denmark, 1994	E	ND
CECT 5198	Diseased eel	Spain, 1999	A	+
CECT 5343	Diseased eel	Spain, 2000	A	+
CECT 5768	Diseased eel	Spain, 2001	A	+
CECT 5769	Diseased eel	Spain, 2002	A	+
A10	Diseased eel	Spain, 2002	A	ND

Strain	Source	Country, yr	Serovar [a]	Virulence for eels
A11	Diseased eel	Spain, 2002	A	ND
A12	Diseased eel	Spain, 2002	A	+
A13	Diseased eel	Spain, 2002	A	ND
A14	Diseased eel	Spain, 2002	A	ND
95-8-6	Diseased eel	Denmark, 1995	O3[±]	+[b]
95-8-7	Diseased eel	Denmark, 1995	O3[±]	+[b]
95-8-161	Diseased eel	Denmark, 1995	O3:O4[±]	+[b]
95-8-162	Diseased eel	Denmark, 1995	O3:O4[±]	+[b]
535	Diseased eel	Sweden	NT	+[b]
536	Diseased eel	Sweden	NT	+[b]
ATCC 33816	Human blood	USA	1	-
CECT 4608	Eel farm water	Spain, 1990	1	-
CECT 4609	Healthy eel	Spain, 1990	1	-
CECT 4869	Diseased eel	Unknown	1	-
CECT 5164	Human blood	USA	1	-
CECT 5165	Sea water	USA	1	-
CECT 5166	Wound infection	USA	1	-
CECT 5167	Human blood	Japan	1	-
CECT 5168	Human blood	USA	1	-
CECT 5169	Human blood	USA	1	-
CECT 529[T]	Human blood	USA	1	-
A2	Diseased eel	Spain, 2000	1	-
An4	Diseased eel	Spain, 2000	1	-
An5	Diseased eel	Spain, 2000	1	-
An6	Diseased eel	Spain, 2000	1	-
An7	Diseased eel	Spain, 2000	1	ND
CG021	Oyster	Taiwan, 1993	1	ND
CG022	Seawater	Taiwan, 1993	1	ND
CG023	Seawater	Taiwan, 1993	1	ND
CG024	Seawater	Taiwan, 1993	1	ND
CG025	Oyster	Taiwan, 1993	1	ND
CG026	Oyster	Taiwan, 1993	1	ND
CG027	Oyster	Taiwan, 1993	1	ND
CG028	Oyster	Taiwan, 1993	1	ND
CG100	Oyster	Taiwan, 1993	1	-
CG106	Oyster	Taiwan, 1993	1	-
CG110	Seawater	Taiwan, 1993	1	-
CG111	Seawater	Taiwan, 1993	1	-
CG118	Seawater	Taiwan, 1993	1	-
CS9133*	Human blood	South Korea	1	-[b]
E-4	Seafood		1	-

Strain	Source	Country, yr	Serovar [a]	Virulence for eels
G-83	Fish	South Korea	1	-
L-49	Brackish water	Japan	1	-
KH-03	Human blood	Japan, 2003	1	-
JE	Oyster	USA	1	- [b]
MLT 362	Environment	USA	1	- [b]
MLT 364	Environment	USA	1	- [b]
MLT 404	Environment	USA	1	- [b]
MLT 406	Environment	USA	1	- [b]
N-87	Human blood	Japan, 1987	1	-
PD-1	Eel tank water	Spain, 2001	1	ND
PD-3	Eel tank water	Spain, 2001	1	ND
PD-5	Eel tank water	Spain, 2001	1	ND
PD-12	Eel tank water	Spain, 2001	1	ND
V1	Eel tank water	Spain, 2001	1	ND
V4	Human blood	Australia	1	-
VV425	Oyster	USA	1	-
VV1003	Environment	USA	1	-
94385	Leg wound	Spain, 2001	1	- [b]
94-9-118	Human disease	Denmark, 1994	1	ND
94-9-119	Human disease	Denmark, 1994	1	ND
94-9-130	Human disease	Denmark, 1994	1	ND
YJ001	Human blood	Taiwan	1	ND
YJ002	Bulla fluid	Taiwan	1	ND
YJ003	Wound infection	Taiwan	1	ND
YJ016*	Human blood	Taiwan	1	ND
YN-03	Human blood	Japan, 2003	1	- [b]
1033	Human disease	Israel, 1996	3	- [b]
11028	Human disease	Israel, 1996	3	- [b]
12	Human disease	Israel, 1996	3	- [b]
162	Human disease	Israel, 1997	3	- [b]
32	Human disease	Israel	3	- [b]
58	Human disease	Israel, 1997	3	- [b]
3/97	Human disease	Israel, 1997	3	- [b]

[a]: Serovars A and E were determined by the serotyping system of Biosca *et al.*, (Biosca, 1994). Serovars O3, O3:O4 and O4 were determined by the serotyping system of Martin and Siebeling NT, non-typable with the antisera against serovars A and E.
B:Data obtained from the present work.
*Strain used as tester for SSH.

Table 10. Other vibrio and related species used in this chapter.

Strain	Source	Country
Aeromonas allosaccharophila CECT 4199^T	Diseased elver	Spain
A. encheleia CECT 4342^T	Healthy elver	Spain
A. hydrophila CECT 839^T	Tin of milk with fishy odor	—
A. jandaei CECT 4338	Diseased elver	Spain
A. sobria CECT 4245 ^{T}	Diseased carp	—
A. jandaei M6	Eel mucus	Spain
Edwarsiella tarda CECT 886	Diseased eel	U.S.A.
Photobacterium damselae ssp. damselae RG191	Internal organ of turbot	Spain
Plesiomonas shigelloides CECT 4354	Liver from healthy eel	Spain
Pseudomonas spp	Diseased tilapia	Spain
Shewanella spp	Diseased tilapia	Spain
V. aesturianus CECT 625^T	Oyster	—
V. algynolyticus CECT 521^T	Horse mackerel	—
Listonella anguillarum 775	Diseased fish	—
V. campbellii CECT 523^T	—	U.S.A.
V. carchariae CECT 4215^T	Kidney of brown shark	U.S.A.
V. cholerae CECT 653	Water	India
V. cincinnatiensis CECT 4216^T	Human blood	U.S.A.
V. diazotrophicus CECT 627^T	Gastrointestinal tract of sea urchin	Canada
V. fischeri CECT 524^T	—	—
V. fluvialis CECT 4217^T	Seawater	Spain
V. furnissii CECT 4203^T	Human faeces	Japan
V. furnissii CECT 4349	Brackish water of an eelfarm	Spain
V. harveyi CECT 605	Marine plankton	U.S.A.
V. mediterranei CECT 621^T	Coastal marine sediment	Spain
V. mimicus CECT 4218^T	Human ear	U.S.A.
V. mytilii CECT 632^T	Mussels	Spain
V. natriegens CECT 526^T	Salt marsh mud	U.S.A.
V. nereis CECT 595^T	Seawater	U.S.A.
V. nigripulchritudo CECT 628T	Seawater	U.S.A.
V. ordalii CECT 582^T	Kidney of coho salmon	U.S.A.
V. orientalis CECT 629^T	Seawater	China

Strain	Source	Country
V. parahaemolyticus CECT 611	—	Spain
V. proteolyticus CECT 630^T	Wood-boring isopod	U.S.A.
V. salmonicida CECT 4195^T	Atlantic salmon	Norway
V. splendidus CECT 528^T	Marine fish	—
V. scophtalmi CECT 4638^T	Turbot	U.S.A.

—, not available.

DNA manipulations.

The genomic DNA of each strain was extracted using standard procedures (Ausubel *et al.*, 1999). Restriction endonuclease digestion was performed as sugested by the manufacturers. Plasmid DNA was prepared with the Minipreps DNA Purification System (Promega, Madison, WI).

Suppression Subtractive Hybridization.

Suppression subtractive hybridization (SSH) between one Serovar E tester (CECT 4602) and three biotype 1 drivers (CS9133, YJ016 and CECT 529) was performed with the PCR-Select Bacterial Genome Subtraction Kit (Clontech, Palo Alto, CA) as described in the user's manual. The PCR products obtained were cloned into PGEM-T easy vector (Promega), transformed into *E. coli* DH5α, and positive clones were selected on LB medium supplemented with ampicillin, X-gal, and IPTG.

Southern Hybridization.

Southern hybridization was performed to check the specificity of the products of SSH, identify the subtractive clones that contain tester-specific DNA fragments, and determine the locations of tester-specific DNA sequences. The probes were labeled with [α-^{32}P]dCTP (Amersham Biosciences, Little Chalfont, Buckinghamshire, England) by random priming using either the PCR products or DNA fragments excised from the recombinant plasmids as templates. Approximately 10 μg of DNA was

fractionated by electrophoresis on a 0.8% agarose gel and transferred to a Hybond-N+ nylon membrane (Amersham Biosciences). The blots were prehybridized for 30 min at 68°C with 5 ml of ExpressHyb Hybridization solution (Clontech), hybridized for 1.5 h at the same temperature, washed and visualized by autoradiography.

DNA sequence analysis.

The nucleotide sequence was determined by an autosequencer (Applied Biosystems PRISM 3700). The determined DNA sequences were further subjected to search for homologous sequences from the GenBank database with the SeqWeb program of Genetics Computer Group provided by National Health Research Institute, Taiwan, R.O.C.

Polymerase chain reaction (PCR).

To check the specificity of the sequences for biotype 2 and/or serovar E, the primer pairs derived from the tester-specific sequences were used in PCR with a wide collection of *V. vulnificus* strains, belonging to the three biotypes and serovars, and other species. To this end, the genomic DNA isolated from an overnight bacterial culture was used as the template. The reaction was composed of 50 μl mixture containing 200 ng DNA, 5 μl of 10× PCR buffer (Amersham Biosciences), 6.25 mM dNTPs, 75 mM $MgCl_2$, 10 μM of each primer and 2.5 U of Taq polymerase (Amersham Biosciences). The PCR started with a 5-min denaturation at 94°C followed by 25 cycles of a 30-sec denaturation at 94°C, an 1-min annealing at 55-65°C (see table 11 for concret annealing temperature), and an 1-min extension at 72°C. An additional extension at 72°C for 10 min completed the reaction.

Table 11. Primer sequences derived from the specific sequences obtained from SSH.

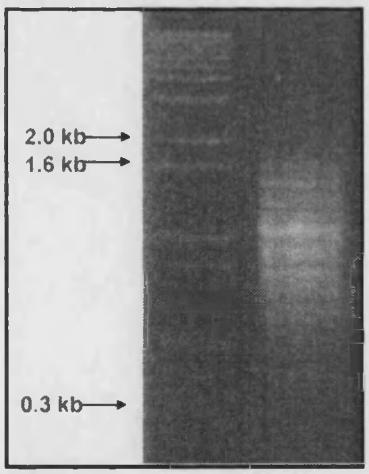
Primer	Derived from (sequence no.)	Sequence (5'-3')	Anneal. T° (C)	Product size (bp)
VF05	05	AACCACATCCAAGACTCTCGCC	62	193
VR05	05	ACTTAAACACCACTGTGCCTCC		
VF10	10	CATCACTCAACTTCTCGACTCC	58.5	384
VR10	10	AGCATCTCACCACGACGAC		
VF12	12	CGTGTTGATTTTATCCGCCTCC	55	466
VR12	12	ACTCTCTCCCGTTATCTGCC		
VF23	23	ACATAAGGGGGACGGAGAG	62	495
VR23	23	CCCCGCCAAAACATAAACAG		
VF25	25	GCCAAGTGCTAATCCATCC	60	411
VR25	25	TGCTCAAAGCCATACTCTCC		
VF51	51	GGACAGATACAAGGGCAAATGG	65	344
VR51	51	AGAGATGGAAGAAACAGGCG		
VF61	61	CGCGCTTAGATTTGTCTCACC	57	665
VR61	61	TGTTGTTCTTGCCCACTCTC		
VF67	67	AAAGCCACACAGACGGAC	60	462
VR67	67	CACCGCTGAATATCACGAG		

RESULTS

Genomic subtraction between biotype 1 and Serovar E *V. vulnificus* strains.

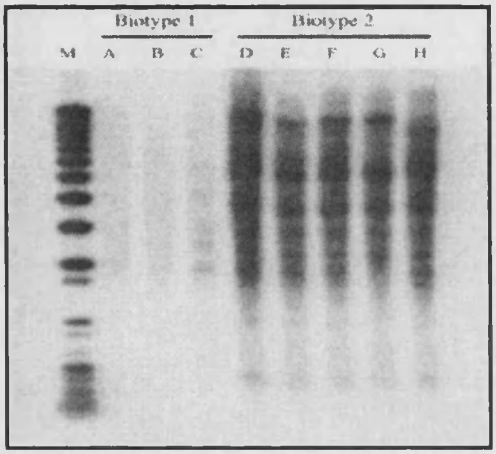
SSH technique was performed with one serovar E strain, CECT 4602, as the tester and three biotype 1 strains, CS9133, YJ016 and CECt 529, as the drivers. The average size of the PCR products was approximately 300 to 1,500 bp (Figure 10). The subtraction products amplified after PCR were shown to hybridize only with the selected serovar E strains, but not with any of the biotype 1 strains tested (Figure 11). The subtractive products were cloned into PGEM-T easy vector, and the specificity of the DNA fragment in each clone was checked by southern hybridization. Among the 85 clones obtained from the subtractive library, eight that did not cross-hybridize with each other and were specific to the selected serovar E strain (see an example in Figure 12) were identified.

Figure 10. PCR products of the SSH.



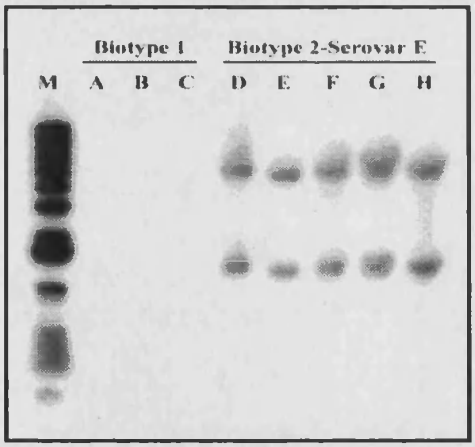
2% Agarose gel , TAE 1X

Figure 11. Southern hybridization of the genomic DNAs of various *V. vulnificus* strains probed with the subtraction products.



*Hind*III digested genomic DNAs were prepared from the biotype 1 strains,; CS9133, CECT 529 and YJ016 (lanes A to C) and biotype 2 serovar E strains, CECT 4602, CECT 4601, CECT 4604, CECT 4605 and ATCC 33147 (lanes D to H) M: 1 kb plus DNA ladder.

Figure 12. Southern hybridization to examine the specificity of cloned sequences for the bt2-SerE strains.



The genomic DNA of biotype 1 strains, CS9133, CECT 529 and YJ016 (lanes A to C), and bt2-SerE strains, CECT 4602, CECT 4601, CECT 4604, CECT 4605, and ATCC 33147 (lanes D to H). They were digested with *Hind*III and then probed with the plasmid DNA extracted from the subtractive clone, CT005. M: 1 kb plus DNA ladder.

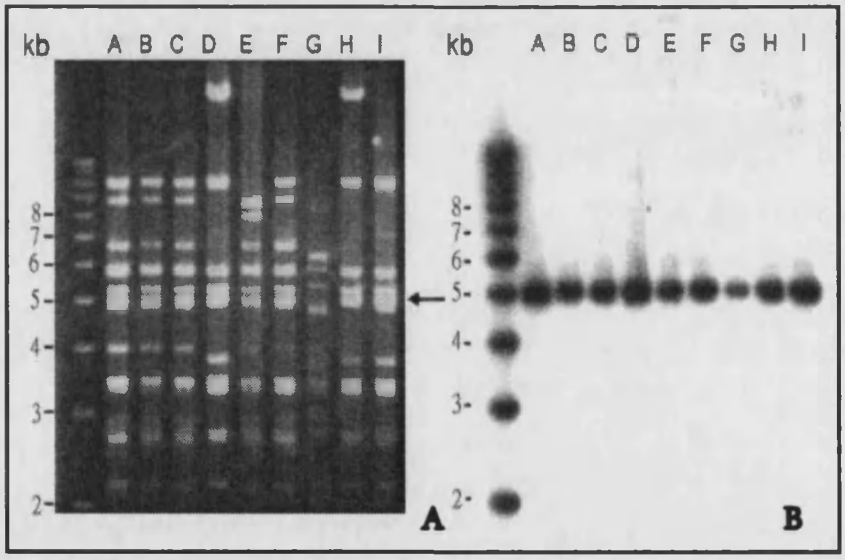
Characterization of the tester-specific regions.

In order to characterize the DNA fragments contained in the eight clones, each clone was subjected to sequence determination and search for homologous sequences. Among the eight tester-specific sequences, four showed significant homology in the amino acid sequence to other database entries as shown in Table 12. Clones CT005 and CT010 both shared high homology with a putative transposase in *Listonella anguillarum*, while CT012 and CT067 were highly homologous to a hypothetical protein in *Aeromonas salmonicida* and *V. parahaemolyticus*, respectively. The location of each cloned DNA fragment was further determined by southern hybridization using total DNA and plasmid DNA, respectively, of strain CECT 4602 as the templates. The DNA sequence was considered to be located in the plasmid if the probe derived from which hybridized with both the plasmid and total DNA. On the contrary, the DNA sequence was considered as being located in the chromosome, if the probe derived from which hybridized with the total DNA, but not with the plasmid. Five of them were found to be located on the plasmid (Table 12). We then examined the plasmid profiles of eight Serovar E and one serovar A biotype 2 strains and found that all of them harboured at least one plasmid. The Serovar E strains showed similar restriction patterns, which were remarkably different from that of the serovar A strain. Nevertheless, all the biotype 2 strains tested, regardless it was serovar E or serovar A, shared a common HindIII-restricted DNA fragment that hybridized with sequence 51, one of the identified tester-specific sequences (Figure 13).

Table 12. Features of the tester-specific DNA sequences in the various subtractive clones

Clone no.	Length (bp)	Homologous protein	Species	*E* value	Location
CT005	291	Putative transposase	*Listonella anguillarum*	1e-41	Plasmid
CT010	1198	Putative transposase	*Listonella anguillarum*	3e-46	Plasmid
CT012	742	Transposase	*Aeromonas punctata*	3e-67	Plasmid
CT023	600	None			Chromosome
CT025	626	None			Plasmid
CT051	1401	None			Plasmid
CT061	805	None			Chromosome
CT067	671	Hypothetical protein	*Vibrio parahaemolyticus*	1e-69	Chromosome

Figure 13. Southern hybridization of plasmids extracted from various biotype 2 strains with a biotype 2-specific sequence.



The plasmid DNA purified from each strain was digested with *Hin*dIII, fractionated by electrophoresis on 0.8% agarose gel, and visualized by staining with ethidium bromide. (b) Southern hybridization of the digested plasmid DNA as shown in (a) probed with the ^{32}P-labeled sequence 51. Lanes A to I: CECT 4601, CECT 4602, CECT 4604, CECT 4605, ATCC 33147, CECT 4603, CECT 5198, CECT 4607 and CECT 4864. The DNA fragment in (a) that hybridized with sequence 51 is indicated by an arrow.

Distribution of the tester-specific sequences among *V. vulnificus* and non-*V. vulnificus* strains.

A collection of 57 biotype 1, 47 biotype 2, and 7 biotype 3 *V. vulnificus* strains, together with 37 non *V. vulnificus* strains were tested for the presence of the identified tester-specific sequences by PCR with the primer pairs showed in Table 11. None of the eel-avirulent *V. vulnificus* strains, including the biotype 1 and biotype 3 strains, gave PCR products with any of the six primer pairs derived from the sequences 10, 23, 25, 51, 61, and 67 (Table 13). Furthermore, all the biotype 2 strains, irrespective of their serovars, gave a positive reaction with the primer pairs derived from the sequences 10, 25 and 51. On the other hand, only the serovar E strains gave positive reactions with the primer pairs derived from sequences 23, 61 and 67 (Table 13). All of the non-*V. vulnificus* strains gave a negative result with all the primer pairs tested. The primer pair derived from sequence 5 not only reacted with all of the biotype 2 strains but also with three biotype 3 strains (VV1033, VV12 and 11028). Finally, the primer pair from sequence 12 was ruled out at the beginning of the study because it gave positive reactions with some biotype 1 strains and negative reactions with several biotype 2 strains.

Eel virulence.

The virulence of the *V. vulnificus* strains in eels is shown in Table 9. As expected, all tested biotype 2 strains, irrespective of the serovar, were virulent for eels and all tested biotype 1 and biotype 3 strains were avirulent.

Table 13. PCR results of V. vulnificus strains tested with some of the primers derived from SSH.

		Primer pair [b]					
Strain	n[a]	10	25	51	23	61	67
Eel-avirulent							
Biotype 1	57	0	0	0	0	0	0
Biotype 3	7	0	0	0	0	0	0
Eel-virulent Biotype 2							
Serovar E	32	32	32	32	32	32	32
Serovar A	9	9	9	9	0	0	0
Serovar O3	2	2	2	2	0	0	0
Serovar O3/O4	2	2	2	2	0	0	0
Other serovars	2	2	2	2	0	0	0

[a]: Total number of tested strains.

[b]: Number of strains that gave a PCR product of the predicted size.

DISCUSSION

The biotype 2 *V. vulnificus* comprises the eel-virulent strains, which are biochemically and serologically heterogeneous (Biosca *et al.*, 1997a; Biosca *et al.*, 1996b; Dalsgaard *et al.*, 1999; Fouz and Amaro, 2003; Høi *et al.*, 1998b; Tison *et al.*, 1982). In spite of this heterogeneity, we hypothesized that the host specificity of the biotype 2 strains for eels might reside in specific DNA regions that are common to all strains of this biotype. By SSH, we obtained a subtractive library of 85 clones, from which only eight sequences were specific to the tester strain. These sequences were shared by four additional serovar E strains, and were selected for further studies. The low frequency of tester-specific DNA fragments suggests that the biotype 1 and biotype 2 strains share high sequence homology in their genomes.

Database searches showed that four (sequences 5, 10, 12, and 67) out of the eight tester-specific DNA sequences, when compared the putative protein sequence, were similar to four hypothetical proteins encoded by the genomes of three pathogenic species, *A. salmonicida, L. anguillarum* and *V. parahaemolyticus*. The other four sequences seem to be unique, since they could not be related to any published sequence, until date. With respect to the localization of the tester-specific sequences, five sequences were located in the plasmids, and the other three in the chromosome. Interestingly, two plasmidic sequences (sequences 05 and 10), when translated, were highly similar to two hypothetical proteins encoded from the virulence plasmid of the fish pathogen *L. anguillarum* (Di Lorenzo *et al.*, 2003). The presence of homologous sequences in the plasmids of two eel pathogens that are not closely related suggests that these sequences may have been transferred horizontally from one organism to the other, maybe when both were colonizing the eels. Transfer of DNA via a bacteriophage between strains that colonize closely in the host tissue has been described in *V. cholerae* (Faruque *et al.*, 1998).

We further designed primer pairs according to the specific sequences, and tested their specificity by PCR with a wide collection of *V. vulnificus* strains of all biotypes, serovars and origins, and with a collection of strains of other species. None of these sequences was present in other species, including *A. salmonicida, L. anguillarum* and *V. parahaemolyticus*. This result demonstrates that although some sequences showed high homology with those of *L. anguillarum* (sequences 5 and 10), *A. salmonicida* (sequence 12), and *V. parahaemolyticus* (sequence 67), respectively, the primer sequences designed were not shared by these species. Consequently, these primers could be useful for the diagnosis of vibriosis. Furthermore, only three sequences (sequences 10, 25, and 51) were present in all biotype 2 strains irrespective of the serovar, and they all were plasmid-borne. It has been previously reported that all the serovar E strains examined harbored plasmids of high molecular weight (Biosca *et al.*, 1997a), suggesting a role of these plasmids in eel virulence. In another study, Lewin *et al.* (2000) demonstrated that the plasmids in serovar E strains were very similar by their restriction patterns and the results of southern hybridization with a plasmidic probe. In this study, we confirmed that the serovar E plasmids are highly homogeneous.

The presence of plasmids in serovar O3 strains had been previously reported by Høi et al (1998b). The plasmids of one serovar A strain was purified and analyzed in this study and we found that although its restriction pattern was significantly different from that of the serovar E strains, it shared at least a common sequence with the serovar E plasmids. These results suggest that the genetic determinants for eel virulence might be encoded by a virulence plasmid, which could have been acquired by an ancestor of *V. vulnificus* biotype 2 from an unknown donor by conjugation. This plasmid could have evolved in different ways to give rise to different plasmids that are related but can be differentiated after digestion by restriction enzymes.

The implication of plasmids in bacterial virulence have been previously reported in other bacterial pathogens such as

Salmonella (Guiney *et al.*, 1995), *Yersinia* (Cornelis *et al.*, 1989), and *L. anguillarum* (Crosa, 1980; Crosa *et al.*, 1980). In the case of *L. anguillarum*, plasmid pJM1 encodes an efficient iron-sequestering system, and cure of this plasmid from the organism significantly reduces its virulence for fish (Crosa, 1980). To determine the linkage of biotype 2 plasmids with bacterial virulence in eels, plasmid curing experiment with some serovar E and non-serovar E strains are in process in our laboratory, and cured strains will be tested for eel virulence.

The exhaustive analysis by PCR also revealed that all serovar E strains shared three sequences that are located on the chromosome. One of them, sequence 67, when translated, was shown to be highly homologous to a hypothetical protein encoded by the chromosome of *V. parahaemolyticus*. It has been demonstrated previously that the non-serovar E strains are less virulent and grow slower in eel serum than the serovar E strains (Esteve-Gassent *et al.*, 2004a; Fouz *et al.*, 2001). It has also been demonstrated that the ability of serovar E strains to grow efficiently in eel serum is conferred by the O-antigen (Amaro *et al.*, 1997). On the other hand, five indole-negative strains (GG100, CG106, CG110, CG111 and CG118) have been isolated from the marine environments of southwestern area in Taiwan (Hor *et al.*, 1995). These strains were avirulent for eels and sensitive to eel serum, although reacted with the anti-serovar E antiserum. In addition, the LPS of these isolates lacked the highest molecular-weight (Mr) portion. From these results we proposed that the highest Mr portion of LPS may be responsible for eel virulence and resistance to eel serum (Amaro *et al.*, 1999). In the present work, we found that these indole-negative Taiwanese isolates did not react with either the biotype 2-specific primer pairs or the serovar E-specific ones. Collectively, these findings suggest that the three serovar E-specific sequences could be involved in the synthesis or modification of high Mr-portion of LPS and may be related to the high virulence of the serovar E strains for eels.

In summary, by using SSH we have identified the DNA sequences of *V. vulnificus* that could be used for distinguishing the eel-virulent strains from non-virulent strains or the serovar E strains from non-serovar E strains. The presence of common biotype 2-specific sequences on the plasmids suggests that the eel virulence of this organism could have been acquired by horizontal transfer of a virulence plasmid. Finally, the sequences identified in this study could be very useful for developing a multiplex PCR method for diagnosis of eel vibriosis caused by *V. vulnificus* and the detection of serovar E, biotype 2 strains (See chapter 5). With this methodology, we could better limit the transmission of serovar E, biotype 2 *V. vulnificus* to humans by rapid detection and proper disposal of the contaminated areas or products.

5

MULTIPLEX PCR ASSAY FOR DETECTION OF *V. vulnificus* BIOTYPE 2

INTRODUCTION

Traditional methods of bacterial identification depend on phenotypic methodologies that involve microscopic staining and growth of bacteria in different culture media to detect physiological requirements and/or biochemical abilities. However, these methods for bacterial identification suffer from major drawbacks. First, they rely on the growth of the bacteria so they can be used only with organisms that can be cultured in vitro. Second, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species. Moreover, these methods need some time, depending on the growing rates of the bacteria, and under a practical approach this identification delay could have negative consequences. When talking about a pathogen a rapid diagnose could be critical for a successful treatment. The use of molecular techniques for the identification of isolates or as diagnostic tools could solve this problems.

The first link between diseased-fish manipulation and human vibriosis caused by *V. vulnificus* was established by Veenstra *et al.* in the Netherlands in 1992 (Veenstra *et al.*, 1992). These authors hypothesized that diseased eels could constitute a risk for Public Health because the fish pathogen *V. vulnificus* biotype 2 could sporadically infect humans. The hypothesis was confirmed after the identification of one human isolate deposited on the American Type Culture Collection (ATCC) as belonging to biotype 2 and serovar E (Amaro and Biosca, 1996). Although new human cases of vibriosis have been reported in northern Europe (Bock *et al.*, 1994; Bruun *et al.*, 1996; Dalsgaard *et al.*, 1996b; Hoyer *et al.*, 1995; Veenstra *et al.*, 1993), none of these isolates has been identified at subspecies level. These cases have been related to an increase in seawater temperature surrounding Baltic countries (water with salinity adequate for *V. vulnificus* survival) due to atypical warm years and some of them were acquired after fish manipulation.

The main objective of the present work was to develop a biotyping procedure based on molecular methods, such us a PCR procedure, that simplifies the identification of the fish pathogen *V.*

vulnificus biotype 2 and, at the same time, allows the discrimination of those isolates with human pathogenic potential (serovar E). A secondary objective was to adapt the PCR protocol to vibriosis diagnostic and sensitive detection of the pathogen in sub-clinical carrier fish. For the design of the PCR primer sets, we selected the cytolisin gene *vvhA*, which is present in all *V. vulnificus* strains regardless the biotype (Wright *et al.*, 1985; Yamamoto *et al.*, 1990), and two DNA sequences specific for biotype 2 and serovar E, respectively, described in the previous chapter.

MATERIALS AND METHODS

Bacterial strains.

To test the specificity of the multiplex PCR, a total of 102 *V. vulnificus* strains (Table 14), most of which previously biotyped, together with 37 strains of other Vibrio species and related genera were used (Table10, chapter 4).

Table 14. *V. vulnificus* used in this chapter.

Strain	Source	Country, yr	Biotyping
535	Diseased eel	Sweden	NT
536	Diseased eel	Sweden	NT
95-8-161	Diseased eel	Denmark, 1995	O3:O4
95-8-162	Diseased eel	Denmark, 1995	O3:O4
95-8-6	Diseased eel	Denmark, 1995	O3
95-8-7	Diseased eel	Denmark, 1995	O3
CECT 5343	Diseased eel	Spain, 2000	A
CECT 5768	Diseased eel	Spain, 2001	A
CECT 5769	Diseased eel	Spain, 2002	A
CECT 5198	Diseased eel	Spain, 1999	A
A10	Diseased eel	Spain, 2002	A
A11	Diseased eel	Spain, 2002	A
A12	Diseased eel	Spain, 2002	A
A13	Diseased eel	Spain, 2002	A
A14	Diseased eel	Spain, 2002	A
90-2-11	Diseased eel	Denmark, 1990	E
CECT 4601	Diseased eel	Spain, 1989	E
CECT 4602	Diseased eel	Spain, 1990	E
CECT 4603	Diseased eel	Spain, 1990	E
CECT 4604	Diseased eel	Spain, 1990	E
CECT 4605	Diseased eel	Spain, 1990	E
CECT 4607	Diseased eel	Spain, 1992	E
CECT 4862	Diseased eel	Japan, 1979	E
CECT 4864	Diseased eel	Spain, 1994	E
CECT 4868	Diseased eel	Norway, 1990	E
CECT 4870	Diseased eel	Sweden, 1991	E
CECT 4917	Diseased eel	Spain, 1997	E
CECT 4998	Diseased eel	Spain, 1997	E
CECT 4999	Diseased eel	Spain, 1999	E

Strain	Source	Country, yr	Biotyping
CECT 5139	Diseased eel	Spain, 1998	E
CECT 897	Diseased eel	Japan, 1979	E
CECT 898	Diseased eel	Japan, 1979	E
Ö122	Diseased eel	Sweden, 1995	E
CECT 4865	Diseased shrimp	Taiwan	E
CECT 5763	Eel tank water	Spain, 2002	E
PD-2-47	Eel tank water	Spain, 2003	E
PD-2-50	Eel tank water	Spain, 2003	E
PD-2-51	Eel tank water	Spain, 2003	E
PD-2-55	Eel tank water	Spain, 2003	E
PD-2-56	Eel tank water	Spain, 2003	E
C1	Healthy eel	Spain, 2003	E
CECT 5762	Healthy eel	Spain, 2002	E
CECT 4866	Human blood	Australia	E
CECT 4863	Leg wound	USA	E
94-9-123	Seawater	Denmark, 1994	E
Riu 2	Seawater	Spain, 2003	E
94-8-112	Wound infection	Denmark, 1994	E
CCUG 38521	Wound infection	Sweden, 1997	ND
CIP 81.90	Human Blood	France, 1980	ND
L-49	Brackish water	Japan	1
A2	Diseased eel	Spain, 2000	1
An4	Diseased eel	Spain, 2000	1
An5	Diseased eel	Spain, 2000	1
An6	Diseased eel	Spain, 2000	1
An7	Diseased eel	Spain, 2000	1
CECT 4869	Diseased eel	Unknown	1
CECT 4608	Eel farm water	Spain, 1990	1
PD-1	Eel tank water	Spain, 2001	1
PD-12	Eel tank water	Spain, 2001	1
PD-3	Eel tank water	Spain, 2001	1
PD-5	Eel tank water	Spain, 2001	1
V1	Eel tank water	Spain, 2001	1
MLT 362	Environment	USA	1
MLT 364	Environment	USA	1
MLT 404	Environment	USA	1
MLT 406	Environment	USA	1
VV1003	Environment	USA	1
G-83	Fish	South Korea	1
CECT 4609	Healthy eel	Spain, 1990	1
ATCC 33816	Human blood	USA	1
CECT 5164	Human blood	USA	1
CECT 5167	Human blood	Japan	1

Strain	Source	Country, yr	Biotyping
CECT 5168	Human blood	USA	1
CECT 5169	Human blood	USA	1
CECT 529^T	Human blood	USA	1
CS9133	Human blood	South Korea	1
KH-03	Human blood	Japan, 2003	1
N-87	Human blood	Japan, 1987	1
V4	Human blood	Australia	1
YJ016	Human blood	Taiwan	1
YN-03	Human blood	Japan, 2003	1
94-9-118	Human disease	Denmark, 1994	1
94-9-119	Human disease	Denmark, 1994	1
94-9-130	Human disease	Denmark, 1994	1
94385	Leg wound	Spain, 2001	1
CG100	Oyster	Taiwan, 1993	1
CG106	Oyster	Taiwan, 1993	1
JE	Oyster	USA	1
VV425	Oyster	USA	1
CECT 5165	Sea water	USA	1
E-4	Seafood		1
CG110	Seawater	Taiwan, 1993	1
CG111	Seawater	Taiwan, 1993	1
CG118	Seawater	Taiwan, 1993	1
CECT 5166	Wound infection	USA	1
1033	Human disease	Israel, 1996	3
11028	Human disease	Israel, 1996	3
Bt3-12	Human disease	Israel, 1996	3
Bt3-162	Human disease	Israel, 1997	3
Bt3-97	Human disease	Israel, 1997	3
Bt3-32	Human disease	Israel	3
Bt3-58	Human disease	Israel, 1997	3

Extraction of DNA from bacterial cultures.

Template DNA was prepared directly from the bacterial culture by the boiling method. Briefly, a 1 ml portion of the culture was centrifuged at 10,000Xg for 10 min. The bacterial pellet was washed once with saline solution (SS) (0.9% NaCl), centrifuged, resuspended in 100 µl of sterile saline solution, boiled for 10 min, and snap-cooled on ice. The lysate was centrifuged at 10,000X g for 10 min and around 1 to 5 µl of the supernatant was used as template for PCR.

Extraction of DNA from eel.

Cross-sectional slice (ca. 1.5 to 2.2 mg) of tissue from liver and kidneys were homogenized in 100 µl of sterile saline solution. Then, 300 µl of lysis solution were added (100 mM Tris HCl, 5 mM EDTA, 200 mM NaCl, 2% SDS, 100 µg proteinase K, pH 8) to the homogenized tissues and the tubes were placed in a 55ºC water bath for 2 hours, followed by a centrifugation at 10,000X g for 5 min to precipitate cell debris. 2V of ice-cold ethanol absolute were added drop by drop to the supernatant, samples were mixed and placed to -20º C for 20 min. DNA was precipitated by centrifugation at 1000 x g for 15 min. The DNA pellet was then washed in 70% ethanol and moderately dried. The dried DNA pellet was resuspended in 30-40 µl of 10 mM Tris-HCl, pH 8.

Polymerase chain reaction (PCR).

Table 15 shows the sequences of the three primer pairs used in this study. PCRs were performed in 50 µl-reaction volumes that contained 1X PCR buffer (Roche Diagnostics), 1.5 mM $MgCl_2$, 200 µmol of each deoxynucleoside triphosphate (Roche), 1.5 U of Taq polymerase (Roche Diagnostics), primer concentrations specified in Table 15, and 2-5 µl of sample (cell lysates or purified DNA). The multiplex PCR was performed on a TC-312 thermal cycler (Techne, Duxford, Cambridge, U.K.) and the selected parameters were: an initial denaturation step at 94ºC for 4 min; followed by 35 serial cycles of 1 min at 94ºC for denaturing, 45 s at 64ºC for annealing, and 1 min at 72ºC for extension; and a final extension step at 72ºC for 10 min. A negative control (no template DNA) and a positive control (2 ng of purified DNA of the biotype 2 serovar E strain CECT 4602) were included in each batch of PCR. The amplified products were separated by electrophoresis on 1.8% agarose gels and were visualized by staining with ethidium bromide.

Table 15. Primer sequences used in the Multiplex PCR

Primer	Sequence (5'→3')	Product size (pb)	Final con (μM)	Source
SE-R	CGCGCTTAGATTTGTCTCACC	665	0,2	(Lee *et al.*, 2005)
SE-F	TGTTGTTCTTGCCCACTCTC			
Bt2-R	GGACAGATATAAGGGCAAATGG	344	0,2	(Lee *et al.*, 2005)
Bt2-F	AGAGATGGAAGAAACAGGCG			
vvhA-R	CCGCGGTACAGGTTGGCGC	519	0,1	(Hor *et al.*, 1995)
vvhA-F	CGCCACCCACTTTCGGGCC			

Use of the Multplex PCR assay with animals.

To adapt the multiplex PCR to fish vibriosis diagnosis, we artificially infected eels by intraperitoneal injection and processed liver and kidney from moribund animals recovered before 48 h post-infection for both microbiological and PCR analysis. In order to test the sensitivity of the assay we contaminated eel tissue with 10-fold dilutions in PBS-1 of an overnight culture in TSB-1 of the biotype 2-serovar E strain CECT 4602 and processed as described above. The Multiplex PCR as diagnostic tool was then tested with eels naturally affected from vibriosis. These eels came from one intensive fishfarm that cultures eels in freshwater (farm A). Internal organs were aseptically taken and processed for microbiologically analysis and DNA extraction as described before.

The multiplex PCR was also used for detection of healthy carriers. In this case, we sampled apparently healthy eels from another local farm that had registered vibriosis 1 year before (farm B). Gills were selected for sampling since the fish pathogen preferentially colonizes this organ (Valiente and Amaro, 2006). Gills were extracted and processed for DNA extraction and PCR analysis as described before. In parallel, gill mucus was sampled with swabs soaked in SEB-1 or APW (Sanjuan and Amaro, 2004). Swabs were incubated in the

same medium for 6 h and DNA was extracted from the cultures (500 µl). The isolation of *V. vulnificus* from enrichment broths, and its identification and biotyping, was performed according to conventional procedures (Cerda-Cuellar *et al.*, 2000; Sanjuan and Amaro, 2004).

RESULTS

Multiplex PCR assay.

With these combination of primers we were able to identified correctly the biotype 2 strains and among them the serovar E ones. No amplicon or faint band of different size were obtained from the strains belonging to the species listed in Table 10 (figure 14B), whereas all *V. vulnificus* strains gave the expected amplification products (Table 16). Specificity of the method, calculated as percentage of coincident results with those produced by the traditional methods was 100%. Two new isolates of human origin, not previously biotyped, presented three bands after PCR amplification (Table 16). The isolates were subjected to the conventional subtyping procedure that involves biochemical and serological identification and virulence tests in eels (Amaro *et al.*, 1995). The isolates CIP 81.90 from human blood (France, 1980) and CCUG 38521 from wound infection (Sweden, 1997) were virulent for eels ($LD_{50}<10^4$ CFU/fish), agglutinated with anti-SerE specific serum, and gave an API20E profile similar to those previously published for BT2-SerE isolates (5106005 and 5306005, respectively).

Table 16. Results of the Multiplex PCR and original biotyping of the strains.

		Original biotyping	n	PCR results		
				SE	VV	BT2
Humans	diseased	Biotype 3	6	-	+	-
	wound	Biotype 1	3	-	+	-
		BT2-SerE	2	+	+	+
		ND	1*	+	+	+
	lung	Biotype 1	1	-	+	-
	blood	Biotype 1	13	-	+	-
		BT2-SerE	2	+	+	+
		ND	1*	+	+	+
Fish	diseased eels	Biotype 1	5	-	+	-
		BT2-SerE	18	+	+	+
		BT2-NoSerE	12	-	+	+
		ND**	8	-	+	+
	healthy eels	Biotype 1	1	-	+	-
		BT2-SerE	2	+	+	+
	diseased shrimps	BT2-SerE	1	+	+	+
	healthy fish	Biotype 1	1	-	+	-
Water	Seawater/	Biotype 1	8	-	+	-
		BT2-SerE	2	+	+	+
	Tank water	Biotype 1	14	-	+	-
		BT2-SerE	6	+	+	+
Seafood		Biotype 1	9	-	+	-

*. New human isolates of biotype 2 and serovar E

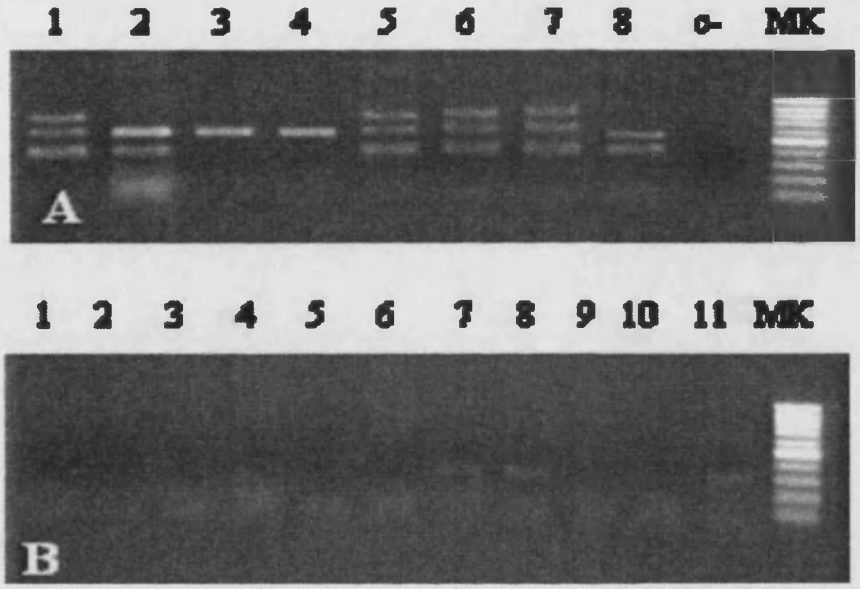
**. Strain isolated from diseased eel from farm A

Use of the multiplex PCR as diagnostic tool.

When the assay was performed with the samples of infected eels, all of them were positive for microbiological and PCR identification (Figure 14). The minimal number of cells that give a clear, positive reaction was 15 CFU/mg tissue. The PCR protocol was then tested with eels naturally affected from vibriosis from the farm A. Four moribund eels were processed for both

microbiological and PCR analysis. In all cases, a biotype 2 non-serovar E amplification profile was obtained from the DNA of the infected tissues. The bacterial isolates were serologically identified with specific antisera as serovar A.

Figure 14. Agarose electrophoresis of the multiplex PCR products obtained for different samples.



Lanes: MK, molecular weight mass DNA ladder low range (Fermentas); (A) Lanes 1 to 4, *V. vulnificus* BT2 Ser E (CECT 4602), BT2 Ser A (CECT 5198), biotype 1 (CECT 529^T) and biotype 3 (BT3-12) control strains; 5, DNA extracted from liver of an eel infected with strain CECT4602; 6, DNA extracted from kidney of an eel infected with strain CECT 4602; 7, DNA extracted from a 6-h culture in APW of gill mucus of an healthy eel from fish farm B; 8, DNA from kidney of a naturally infected eel from fish farm A; 9, negative control (no DNA). (B) Lanes 1, *V. parahaemolyticus*; 2, *V. algynoliticus*; 3, *V.f ischeri*;4, *V. nigripulchitudo*; 5, *V. fluvialis*; 6, *V. mimicus*; 7, *V. furnisii*; 8, *A. hydrophila*; 9, *A. jandaii*; 10, *E. tarda*; 11, *Sh. spp.*

Use of multiplex PCR to detection of healthy carriers.

Serovar E strains was detected by multiplex PCR from 2 gill samples out of 10 healthy eels analyzed, but only after incubation in the appropriate enrichment broth. The same PCR-positive samples were also positive for the isolation of the pathogen,

confirming the feasibility of the protocol for carrier detection. The eels came from a fish farm that had suffered recurrent vibriosis due to serovar E from 1991 to 1998. From that year eels were vaccinated against vibriosis with Vulnivaccine (Collado *et al.*, 2000; Esteve-Gassent *et al.*, 2004a; Esteve-Gassent *et al.*, 2004b; Fouz *et al.*, 2001) and the disease was definitely controlled.

DISCUSSION

In a previous work (see chapter 4), we compared the genomic DNA of a serovar E isolate with the genomic DNAs of three biotype 1 strains by suppression subtractive hybridization and we identified three plasmid-borne DNA sequences that were specific for biotype 2 strains irrespective of the serovar and three chromosomal DNA sequences that were specific for serovar E strains (Lee *et al.*, 2005). The biotype 2-specific sequences could be considered virulence markers since they are present only in the fish virulent strains (Lee *et al.*, 2005). From these sequences we selected two DNA sequences specific for biotype 2 and serovar E, respectively. To complete the assay we included a species specific pair and we selected the cytolisin gene *vvhA*, which is present in all *V. vulnificus* strains regardless the biotype and virulence (Wright *et al.*, 1985; Yamamoto *et al.*, 1990).

When applied the multiplex PCR assay to the previously identified strains, all expected amplicons were obtained. Interestingly, two new isolates of human origin, not previously biotyped, presented three bands after PCR amplification (Table 14). The isolates were subjected to the conventional subtyping procedure that involves biochemical and serological identification and virulence tests in eels (Amaro *et al.*, 1995). The results confirmed that isolates CIP 81.90 from human blood (France, 1980), and CCUG 38521 from wound infection (Sweden, 1997), belonged to biotype 2 and serovar E since they were; virulent for eels ($LD_{50}<10^4$ CFU/fish), agglutinated with anti-serovar E specific serum, and gave an API20E profile similar to those previously published for serovar E isolates (5106005 and 5306005, respectively). No information about the source of infection is reported for strain CIP 81.90, but in case of the strain CCUG 38521, the patient was wounded with a fishhook while fishing. The rest of human strains identified as serovar E in previous works (Amaro and Biosca, 1996; Biosca *et al.*, 1997a; Lee *et al.*, 2005) came from Australia, Denmark and USA, and only that from Denmark was

clearly related to eel manipulation. Our results suggest that seawater or other fish species apart from eels should be considered as putative reservoirs for this pathogen.

The rapid development of molecular biological techniques offers significant advantages for the disease diagnosis, among them the PCR assay is the most commonly used. For these reason, we extend the use of the multiplex PCR assay for the diagnosis of the eel vibriosis. With the PCR we could detect as near 10 CFU/mg tissue that is similar to the detection limits described by others authors (Del Cerro *et al.*, 2002; Mata *et al.*, 2004). While we were performing this study a vibriosis was suspected to outbreak in an eel farm. We took eel samples and performed the multiplex PCR assay in parallel with the traditional diagnosis methods. The results from both methodologies confirmed that eels suffered vibriosis due to a *V. vulnificus* biotype 2 non-serovar E and the serology tests identified the isolates as belonging to serovar A. Thus, the PCR protocol would allow the diagnosis of natural vibriosis from eel tissues in less than 5 h from the point of DNA extraction to observation in an agarose gel.

The PCR assay was also applied to healthy eels from another eel farm. The farm had suffered from recurrent vibriosis between 1991 and 1998, but currently the disease is controlled by vaccination with Vulnivaccine, a vaccine designed against serovar E. Vaccinated eels present specific antibodies in serum against serovar E cells and are well protected against the disease (Esteve-Gassent *et al.*, 2004a; Esteve-Gassent *et al.*, 2004b; Fouz *et al.*, 2001). Interestingly, we obtained some positive results but only after performing an enrichment step. The positive samples came from gills; the organ preferentially colonized by serovar E cells. This result suggests that the pathogen is able to survive and persist in the farm, preferentially associated to immunized eels, and is in accordance with previous studies that suggested that survivors after vibriosis can act as carriers (Valiente and Amaro, 2006). Thus, the PCR protocol would allow detection of carriers without killing the

animals. In this case, PCR would be performed after enrichment in APW or ESB for 6 h.

In conclusion, the multiplex PCR developed in the present work is a useful tool to detect and identify the fish pathogen *V. vulnificus* biotype 2 from multiple types of samples. The assay would allow discrimination of those pathological cases that constitute a risk to Public Health. In addition, the multiplex assay could be used in epidemiological studies to correctly biotype clinical isolates and clarify the status of serovar E as human pathogen.

6

GENOTYPIC AND PHENOTYPIC DIVERSITY OF *V. vulnificus*

INTRODUCTION

As has been described, *V. vulnificus* is a heterogeneous bacterial species that comprises virulent and avirulent strains from environmental and clinical sources. Originally the species was divided into two biotypes;the first is virulent for human and the second for fish. (Tison *et al.*, 1982). Some phenotypic traits, such as negative indole production, ornitine descarboxylase activity, acid production from mannitol and sorbitol, and growth at 42ºC, were found to be useful biotyping criteria for the fish virulent strains. Serologically, all these strains also expressed a homogeneous LPS O-antigen, which was designated serovar E (Biosca *et al.*, 1997a). However, this simple scheme of intraspecific classification lost its utility when more eel pathogenic strains were found. These new isolates presented more phenotypic and serological variability and only eel virulence remained as a distinguishing trait (Dalsgaard *et al.*, 1999; Fouz *et al.*, 2006; 1998b; Høi *et al.*, 1998a). Today, two other serogroups, serovars A and I, have been found within biotype 2 (Biosca *et al.*, 1997a; Fouz *et al.*, 2006; Fouz *et al.*, 2007). In addition to this heterogeneicity found in *V. vulnificus*, a third biotype was described in 1999. To date, this last biotype only includes isolates from human wound infection after fish manipulation in Israel (Bisharat *et al.*, 1999; Colodner *et al.*, 2002).

Due to the Public Health importance of this species and the difficulties for in rapid differentiation of the virulent strains, numerous studies have been conducted in order to clarify the genetic structure of *V. vulnificus* species. Using these methods, some DNA sequence polymorphisms at individual loci appear able to divide *V. vulnificus* populations into two major groups that correlate with the origin of the isolate (environmental vs. clinical) but not with the biotype. Some of these polymorphisms have an epidemiological value since one of the genotypes described is found among the clinical isolates, suggesting a possible correlation between that genotype with human virulence (DePaola *et al.*, 2003; Rosche *et al.*, 2005; Senoh *et al.*, 2005; Warner and Oliver, 1999).

Other genetic typing techniques used for *V. vulnificus* include randomly amplified polymorphic DNA (RAPD), REP-PCR or ribotyping (Arias *et al.*, 1998; Chatzidaki-Livanis *et al.*, 2006; Høi *et al.*, 1997; Tamplin *et al.*, 1996). The majority of these studies were performed with collection of strains biased to biotype 1, since they do not contain biotype 2 and 3 strains, and to North America (Chatzidaki-Livanis *et al.*, 2006; Tamplin *et al.*, 1996), since the majority of the isolates were from that specific geographical region (Chatzidaki-Livanis *et al.*, 2006; Tamplin *et al.*, 1996). On the other hand, Gutaker *et al.* (Gutacker *et al.*, 2003) used other approaches to evaluate the intra-specific diversity of *V. vulnificus*. These authors found that the strains were prone to be grouping into two major divisions, which had no correlation with biotypes or origin of the isolates. However, their phylogenic construct presented several inconstancies. In conclusion and despite all of these studies, the structure of the species and the relationships within and among biotypes remains unclear.

Given this scenario the main objectives of this chapter were i) to evaluate three rapid systems for *V. vulnificus* identification and biotyping and, at the same time, to use them for studying the phenotypic diversity of the species; ii) to validate the potential genotypic markers for human virulence, iii) to validate the ribotyping as a subtyping system, and iv) to find, if possible, some phenotypic or genotypic markers that supported the actual intraspecific classification into biotypes.

MATERIAL AND METHODS

Bacterial strains and growth conditions.

A total of 111 *V. vulnificus* strains of different biotypes, sources and origins were used in this study (Table 17). Strains were grown in Luria broth (LB) or L-agar supplemented with 0.5% (wt/vol) NaCl (LA-1) at 28ºC for 24 h.

Table 17. *V. vulnificus* strains used in this chapter

Strain	MULTIPLEX	DNA polymorphism				Rt
		C/E	*vvhA*	16s rRNA	Profile	
CECT 4869	BT1/3	E	2	A	2	1
CG106	BT1/3	C	1	B	1	2
CECT 5168	BT1/3	C	1	B	1	3
N87	BT1/3	C	1	B	1	3
YJ106	BT1/3	C	1	B	1	3
CECT 5167	BT1/3	C	1	B	1	4
MLT 362	BT1/3	C	1	B	1	5
VV 425	BT1/3	E	1	A	atypical	6
ATCC 33816	BT1/3	C	1	B	1	7
CG110	BT1/3	C	1	B	1	7
CG118	BT1/3	C	1	B	1	8
E4	BT1/3	C	1	B	1	9
CG111	BT1/3	C	1	B	1	10
MLT 364	BT1/3	C	1	B	1	11
VV 1003	BT1/3	C	1	B	1	11
95-8-7	BT2-nonSerE	E	2	A	2	11
CS9133	BT1/3	C	1	B	1	12
CECT 4608	BT1/3	C	1	B	1	13
KH03	BT1/3	C	1	B	1	14
CECT 4862	BT2-SerE	E	2	A	2	14
CECT 5164	BT1/3	C	2	B	atypical	15
Riu-3	BT1/3	E	2	A	2	16
Riu-1	BT1/3	E	2	AB	atypical	16
94385	BT1/3	E	2	B	atypical	17

Strain	MULTIPLEX	DNA polymorphism				Rt
		C/E	*vvhA*	16s rRNA	Profile	
V4	BT1/3	C	1	B	1	18
PD-2-52	BT2-nonSerE	E	2	A	2	19
PD-2-58	BT2-nonSerE	E	2	A	2	20
CECT 4917	BT2-SerE	E	2	A	2	21
CECT 4998	BT2-SerE	E	2	A	2	21
JE	BT1/3	E	2	B	atypical	21
CECT 5165	BT1/3	E	2	A	2	22
A2	BT1/3	E	2	A	2	23
An4	BT1/3	E	2	A	2	23
An5	BT1/3	E	2	A	2	23
An6	BT1/3	E	2	A	2	23
CECT 4606	BT1/3	E	2	A	2	23
PD-1	BT1/3	E	2	A	2	23
PD-12	BT1/3	E	2	A	2	23
PD-3	BT1/3	E	2	A	2	23
PD-5	BT1/3	E	2	A	2	23
V1	BT1/3	E	2	A	2	23
CECT 4605	BT2-SerE	E	2	A	2	23
BT3-11028	BT1/3	E	1	AB	3	24
BT3-162	BT1/3	E	1	AB	3	24
BT3-97	BT1/3	E	1	AB	3	24
BT3-vv12	BT1/3	E	1	AB	3	24
BT3-vv32	BT1/3	E	1	AB	3	24
CECT 5169	BT1/3	C	1	B	1	25
94-9-119	BT1/3	E	2	A	2	25
CECT 4867	BT1/3	E	2	A	2	25
YN03	BT1/3	E	2	A	2	25
535	BT2-nonSerE	E	2	A	2	25
536	BT2-nonSerE	E	2	A	2	25
960426-1/4C	BT2-nonSerE	E	2	A	2	25
960717-1/2F	BT2-nonSerE	E	2	A	2	25
A10	BT2-nonSerE	E	2	A	2	25
A11	BT2-nonSerE	E	2	A	2	25
A13	BT2-nonSerE	E	2	A	2	25
A14	BT2-nonSerE	E	2	A	2	25
CECT 5198	BT2-nonSerE	E	2	A	2	25
CECT 5689	BT2-nonSerE	E	2	A	2	25
CECT 5768	BT2-nonSerE	E	2	A	2	25
CECT 5769	BT2-nonSerE	E	2	A	2	25

Strain	MULTIPLEX	DNA polymorphism				Rt
		C/E	*vvhA*	16s rRNA	Profile	
90-2-11	BT2-SerE	E	2	A	2	25
94-8-112	BT2-SerE	E	2	A	2	25
94-9-123	BT2-SerE	E	2	A	2	25
C1	BT2-SerE	E	2	A	2	25
CECT 4602	BT2-SerE	E	2	A	2	25
CECT 4603	BT2-SerE	E	2	A	2	25
CECT 4604	BT2-SerE	E	2	A	2	25
CECT 4864	BT2-SerE	E	2	A	2	25
CECT 4868	BT2-SerE	E	2	A	2	25
CECT 4870	BT2-SerE	E	2	A	2	25
CECT 5762	BT2-SerE	E	2	A	2	25
CECT 898	BT2-SerE	E	2	A	2	25
CIP 81.90	BT2-SerE	E	2	A	2	25
G83	BT1/3	E	1	B	atypical	25
VV 352	BT1/3	E	1	A	atypical	25
MLT 406	BT1/3	E	2	A	2	26
95-8-6	BT2-nonSerE	E	2	A	2	26
CECT 529^T	BT1/3	E	1	A	atypical	26
CECT 4174	BT2-SerE	E	2	A	2	27
CG100	BT1/3	C	1	B	1	28
L49	BT1/3	E	2	A	2	29
CECT 4607	BT2-SerE	E	2	A	2	30
CECT 4999	BT2-SerE	E	2	A	2	30
PD-2-66	BT1/3	E	2	B	atypical	30
CECT 4601	BT2-SerE	E	2	A	2	31
94-9-130	BT1/3	E	2	A	2	32
CECT 7029	BT2-nonSerE	E	2	A	2	32
CECT 7030	BT2-nonSerE	E	2	A	2	32
95-8-162	BT2-nonSerE	E	2	A	2	33
CECT 4863	BT2-SerE	E	2	A	2	34
CECT 897	BT2-SerE	E	2	A	2	35
95-8-161	BT2-nonSerE	E	2	A	2	36
CECT 5343	BT2-nonSerE	E	2	A	2	36
MLT404	BT1/3	E	2	A	2	37
CECT 4865	BT2-SerE	E	2	A	2	38
CECT 5139	BT2-SerE	E	2	A	2	38
CECT 4866	BT2-SerE	E	2	A	2	39
UE516	BT2-SerE	E	2	A	2	40
94-9-118	BT1/3	E	2	A	2	41

Strain	MULTIPLEX	DNA polymorphism				Rt
		C/E	*vvhA*	16s rRNA	Profile	
534	BT1/3	E	2	A	2	42
PD-2-47	BT2-SerE	E	2	A	2	43
PD-2-51	BT2-SerE	E	2	A	2	43
CECT 5763	BT2-SerE	E	2	A	2	44
PD-2-50	BT2-SerE	E	2	A	2	45
PD-2-55	BT2-SerE	E	2	A	2	45
Riu-2	BT2-SerE	E	2	A	2	46
PD-2-56	BT2-SerE	E	2	A	2	47
CECT 5166	BT1/3	E	2	B	atypical	ND

Phenotypic analysis.

API 20E and API 20 NE test

API 20E and API 20NE test kits (bioMerieux, Paris, France) were used following the manufacturer's direction, incubating the strips at 28ºC. Bacterial suspensions in saline solution (0.9 % NaCl) or in AUX Medium plus NaCl at a final concentration 1% (wt/vol) were used as inoculuma medium for API 20E and API 20NE, respectively (Biosca *et al.*, 1993a). Examination of the strips was conducted after 24 and 48 h. API profiles were compared with the API DataBase version 4.0 for API 20E and version 6.0 in the case of API 20NE (APILAB Software, version 3.3.3, Apilab Plus; Biomerieux)

Biolog

Biolog-GN MicroPlates were used to evaluate the substrate utilization patterns of the *V. vulnificus* strains. The bacteria were streaked on Biolog Universal Growth agar (Biolog) supplemented with 5% sheep erythrocytes (Oxoid) (BUG-S) and incubated for 24 hours at 28ºC.Wells of the plate were inoculated with 150 µl of the bacterial suspension in SS adjusted to the appropriate density. The

inoculated microplates were incubated 28ºC for 24 and 48 h and the plates were analyzed using a Biolog Microstation reader. Identification (Biolog Microlog 6.01 database) and test results were performed using Biolog MicroLog 3 software (Biolog, INC., Hayward, CA, USA), applying the automatic threshold option. A discriminant analysis was performed with the data using SPSS 14.0 for Windows

Genetic fingerprinting.

DNA sequence polymorphism study

The polymorphisms at selected loci were determined by PCR in all *V. vulnificus* strains. Discrimination of biotype 2 together with serotyping (serovar E *versus* non-E) was assessed using the multiplex PCR described in chapter 5 (Sanjuan and Amaro, 2007). Determination of the Environmental- *versus* Clinical- (E- *versus* C-), *vvhA* (haemolysin/cytolysin)-1 *versus* *vvhA*-2 and 16s rRNA-A *versus* -B *versus* -AB genotype was performed as described by Rosche et al (2005) and Senoh et al (2005), respectively. In all the assays, ca. 250 ng of DNA per 25 µl of reaction was amplified using the High Fidelity Expand PCR system (Roche Diagnostics) in a TC-312 thermal cycler (Techne, Duxford, Cambridge, U.K.). Existence of an association between polymorphism and group (biotype, origin or serovar) was calculated using the Pearson Chi-square test function at $\alpha = 0.05$ with SPSS 14.0 for Windows.

Ribotyping.

Ribotyping of the *V. vulnificus* isolates was carried out with the Riboprinter (Qualicon Inc., Wilmington, Del.). The assay was performed under the conditions recommended by the manufacturer except that *Eco*RI was replaced by *Hind*III (Roche) at 400 U/µl in standardized reagents. This enzyme was selected because it provides better discrimination among *V. vulnificus*

isolates and biotypes (Dalsgaard *et al.*, 1996b; Høi *et al.*, 1997). Each strain was cultured onto LA-1, whit one colony then picked, and resuspended in sample buffer and added to the module for heat treatment at 80ºC for 10 min. Then, lytic enzymes were added and samples loaded into a carrier into the instrument where bacterial digestion was performed. Samples were then loaded onto agarose gel cassettes containing 13 wells, five of which were filled with molecular size standard DNA. After electrophoresis, DNA fragments were electroblotted onto a nylon membrane, hybridized with a sulphonated *E. coli rrnB* rRNA operon probe, detected with alkaline phosphatase-labelled antisulphonated-DNA antibodies plus a chemiluminescent substrate (Bruce, 1996) and photographed with a customized camera. Riboprinter patterns were partially processed by the Riboprinter system software in order to reduce background noise and to normalize the band positions using the DNA size standards as references. The normalized patterns were exported for analysis in .txt files and imported into the bionumerics software ver 4.0 (Applied maths, Austin, TX, USA) using the LoadSamples script (DuPont Qualicon). Clustering analysis was performed with the unweighted pair group method using arithmetic average (UPGMA) based on the Dice coefficient for the band matching, with a position tolerance and an optimization setting of 1%. Bands for the band matching were assigned automatically and manually edited if necessary.

RESULTS

Phenotypic analysis.

API20E and API20NE

The coded API 20E and API 20NE profiles generated by the *V. vulnificus* strains and their identification by the API software are shown in Table 18 and 19. In the case of the API 20E system, nearly 60% of the strains were correctly identified as *V. vulnificus,* but the percentage that fell to 20% in the case of the biotype 3. The rest of the strains gave a mixed profile or were misidentified as *Burkholderia cepaciae* or *V. parahaemolyticus.* In the case of the API 20NE system, none of the strains was correctly identified as *V. vulnificus.* Instead of it, most of the isolates were identified at the genus level as *Aeromonas* or *Vibrio,* with the species *A. hydrophila* and *V. cholerae* being the most frequent options (Table 19). One unacceptable API 20 NE profile was obtained from one environmental strain (PD-2-66) that was identified as *Bl. cepacia* with the other API system. Finally, no specific API20E or API20NE profile was associated with biotype, or within biotype 2 to serovar, although all biotype 2 and serovar E strains were negative for the indole test.

Table 18. API 20E profiles obtained.

Profile	Nº of isolates	Identification by API software
4006005	2	*Burkholderia cepacia* (94.6%)
4106005	1	*V. vulnificus* (48.8%) / *Bl. Cepacia* (47.1%)
4146004	2	*V. parahaemolyticus* (85.5%) / *V. vulnificus* (8.7%) / *V. mimicus* (5%)
4146005	1	*V. vulnificus* (83.8%) / *V. parahaemolyticus* (14%)
4306005	3	*Bl. cepacia* (88.8%) / *V. vulnificus* (8.9%)
4346004	1	*V. parahaemolyticus* (90.3%)
5006005	13	*V. vulnificus* (54.4%) / *Bl. cepacia* (45.3%)
5046005	1	*V. vulnificus* (99.9%)*
5046025	1	*V. vulnificus* (76.8) / *A. hydrophila* (20.8%) / *V. cholerae* (2.1%)
5046105	3	*V. vulnificus* (99.5%)*
5106005	5	*V. vulnificus* (98%)*
5144105	1	*V. vulnificus* (99.3%)*
5146005	5	*V. vulnificus* (99.9%)*
5146105	16	*V. vulnificus* (99.7%)*
5206004	1	*Bl. cepacia* (99.5%)
5206005	8	*Bl. cepacia* (89.3%) / *V. vulnificus* (10.4%)
5246104	1	*V. mimicus* (77.2%) / *V. vulnificus* (15.7%) / *V. cholerae* (5.5%) / *A. hydrophila* (1.4%)
5246105	2	*V. vulnificus* (96.4%)*
5306005	7	*V. vulnificus* (83.3%) / *Bl. cepacia* (16.5%)
5306105	5	*V. vulnificus* (98.8%)*
5346005	10	*V. vulnificus* (99.9%)*
5346025	2	*V. vulnificus* (57.6%) / *V. cholerae* (16.5%)
5346105	18	*V. vulnificus* (98.9%)*
5346125	2	*V. cholerae* (88.8%) / *V. vulnificus* (10.7%)

*. Correct identifications

Table 19. API 20NE profiles obtained.

Profile	Nº of isolates	Identification by API software
5450744	1	*A. salmonicida* (83.3%) / *V. alginolyticus* (16.1%)
5450745	2	*A. salmonicida* (83.3%) / *V. alginolyticus* (16.1%)
5462345	1	Unacceptable profile
5472645	1	*V. vulnificus* (75%) / V. cholerae (17.4%) / *V. alginolyticus* (4.6%)
5472744	3	*V. alginolyticus* (83.1%) / *A.hydr*ophila (12.1%)
5472745	28	*V. cholerae* (45%) / *A. hydrophila* (41.7%)
5473745	1	*A. hydrophila/caviae* (94.5%)
5476744	2	*A. hydrophila/caviae* (63.8%) / *V.alginolyticus* (25.6%)
5476745	4	*A. hydrophila/caviae* (86.1%)
6472644	1	*V. alginolyticus* (96.6%)
7062745	2	*V. cholerae* (98.6%)
7070745	1	*V. cholerae* (99.9%)
7072644	1	*V. parahaemolyticus* (51.9%) / *V. cholerae* (33.7%) / *V. alginolyticus* (19.2%)
7072745	1	*V. cholerae* (99%)
7446745	1	V. *alginolyticus* (86.7%)
7462745	1	*V. cholerae* (81%) / *A. hydrophila* (11.5%) / *V. alginolyticus* (6.2%)
7470645	1	*V. vulnificus* (71.3%) / *V. cholerae* (2.8%) / *V. parahaemolyticus* (1.6%)
7472744	6	*V. alginolyticus* (87.4%) / *A. hydrophila* (8.1%) / *V. cholerae* (2.8%) / *V. parahaemolyticus* (1.5%)
7471745	7	*A. hydrophila* (90%)
7474745	1	*V. cholerae* (86.7%)
7476644	1	*V. alginolyticus* (69.5%) / *V. parahaemolyticus* (26.2%) / *A. hydrophila/caviae* (2.4%) / *V. cholerae* (1.1%)
7476744	9	*A. hydrophila/caviae* (54.1%) / *V. alginolyticus* (34%) / *V. parahemolyticus* (10.1%)
7476745	37	*A. hydrophila* (46.8%) / *V. cholerae* (46.3%)
7477745	1	*A. hydrophila* (91.5%) / *V. parahaemolyticus* (8.2%)

Biolog GN2 plates

From the 111 strains studied, Biolog correctly identified 93 isolates (84 %) while misidentifing the reminder. Eight of the misidentifications (7 % of the total strains) led to *V. vulnificus* as the first option although with a low probability. The number of carbon substrates utilized by *V. vulnificus* was 32 as promedium and there were no differences in the number of carbon substrate utilized between the biotypes or origin of the isolates. The use of the different carbon sources of the Biolog GN plate is summarized in table 20. *V. vulnificus* strains were able to use a great variety of different carbon sources with the exception of the amines, which were negative in all cases and carboxylic acids also negative except D-glucoronic and succinic acid were used by more than the 80% of the strains (Table 20). There was no specific profile (expressed as the carbon sources utilized by an isolate) that could be assigned to a particular biotype or serovar, in fact only two strains (PD-1 and PD-5) used the same carbon sources.

Table 20. Percentage of carbon sources used by the *V. vulnificus* collection.

C sources (% used)		
CARBOHYDRATES	**CARBOXYLIC ACIDS**	**AMINO ACIDS**
N-Acetyl-D-galactosamine (72)	Acetic acid (37.5)	D-Alanine (16.2)
N-Acetyl-D-glucosamine (96.4)*	*Cis*-Aconitic acid (9)	L-Alanine (65.7)
Adonitol (0)	Citric acid (0)	L-Alanyl-glycine
L-Arabinose (0)	Formic acid (0)	L-Aspargine (96.4)
D-Arabitol (0)	D-Galactonic acid lactone (0)	L-Aspartic acid (86.5)
Cellobiose (87.4)*	D-Galacturonic acid (1.8)	L-Glutamic acid (91.9)
i-Erythritol (0)	D-Gluconic acid	Glycil-L-aspartic acid (73)
D-Fructose (96.4)		

L-Fucose (0)
D-galatose (54)
Gentibiose (85.6)*
α-D-Glucose (100)*
m-Inositol (0)
α-D-Lactose (36.6)
Lactulose (4.5)
Maltose (89.2)*
D-Mannitol (36)
D-Mannose (87.4)*
D-Melibiose (3.6)
β-Methyl-D-glucoside (58.6)
D-Psicose (35.1)
D-Raffinose (0)
L-Rhamnose (0)
D-Sorbitol (7.2)
Sucrose (6.3)
D-Trehalose (97.3)*
Turanose (4.5)
Xylitol (0)

ESTERS
Mono-methylsuccinate (76.6)
Methylpyruvate (87.4)*

POLYMERS
Glycogen (100)*
α-Cyclodextrine (6.3)
Dextrin (100)*
Tween 40 (84.7)
Tween 80 (92.8)

(33.3)
D-Glucosaminic acid (0)
D-Glucoronic acid (89.9)
α-Hydroxybutiric acid (1.8)
β-Hydroxybutiric acid (0)
γ-Hydroxybutiric acid (0)
ρ-Hydroxyphenylacetic acid (19.8)
Itaconic acid (0)
α-Ketobutyric acid (1.8)
α-Ketoglutaric acid (31.5)
α-Ketovaleric acid (0)
D,L-Lactic acid (58.6)
Malonic acid (0)
Propionic acid (11.7)
Quinic acid (0)
D-Saccharic acid (0)
Sebacid acid (0)
Succinic acid (83.8)

ALCOHOLS
2.3-Butanedio l (0)
Glycerol (56.8)

AMIDES
Succinamic acid (6.3)
Glucuronamide (31.5)
Alaninamide (21.6)

Glycil-L-glutamic acid (31.5)
L-Hystidine (0)
Hydroxy-L-proline (0)
L-Leucine (0)
L-Ornithine (2.7)
L-Phenylalanine (0)
L-Proline (46.8)
L-Pyroglutamic acid (0)
D-Serine (0)
L-Serine (34.2)
L-Threonine (37.9)
D,L-Carnitine (0)
γ-Aminobutyric acid (0)

AROMATIC CHEMICALS
Inosine (91)
Urocanic acid (0)
Thymidine (30.6)
Uridine (72)

BROMINATED **CHEMICALS**
Bromosuccinic acid (51.3)

AMINES
Phenylethylamine (0)
2-Aminoethanol (0)
Putrescine (0)

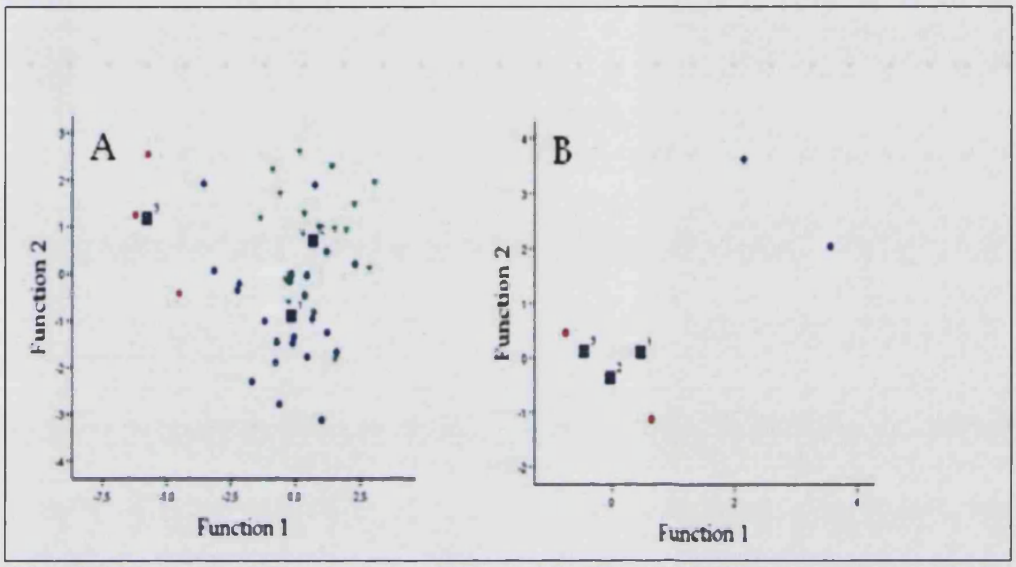
	PHOSPHORYLED CHEMICALS D,L-α-Glycerol phosphate (47.7) Glucose-1-phosphate (79.3) Glucose-6-phosphate (95.5)	

Table modified from Garland et al (Garland and Mills, 1991)
*Alsina's *V. vulnificus* specific test (Alsina and Blanch, 1994a, b)
Range of strains that use that specific carbon source: ■ 0-10%. ■ 10-40% ■ 40-70% ■ 70-90 ■ 90-100

To develop the most complete vision of the phenotypic structure of the *V. vulnificus* population, we subdivided the *V. vulnificus* collection into biotypes (including or not the biotype 3), into origins (environmental//clinical or environmental/human/ veterinary) and into serovars within biotype 2 (serovar E/non-E) and performed a discriminant analysis using the test data after 24-48 hours of incubation. The discriminant analysis generated one (or more) function(s) that provided the best discrimination between different groups of strains. We used this approach to study which carbon sources could characterize the different groups, in order to evaluate the use of the different canonical variable that constituted each discriminant function. In table 21 the carbon sources that have a different percentage of use between the groups studied are summarized. The representation of the two first functions for the *V. vulnificus* collection grouped on their biotype (A) or origin (B) is shown in Figure 15. A combination of five tests (cellobiose, β-methyl-D-glucoside, D-lactose, DL-lactic acid, and glycil-L-aspartic acid) would have biotype-discriminating potential, while a combination of two tests (D-mannitol and DL-α-glycerolphosphate) would help in the discrimination between non-serovar E and serovar E isolates within biotype 2 (Table 21 and Figure 15). No phenotypic test would discriminate strains as regards their origin, although the use of the β-methyl-D-glucoside by the isolates from veterinary source was slightly higher. This difference was highlighted when only biotype 2 strains were considered.

Finally, sucrose, although without significant discriminating potential, was used only by environmental isolates.

Figure 15. Individual scores obtained for the V. vulnificus isolates for the two first discriminant functions.



***V. vulnificus* grouped into biotypes (A) or origin (B).** (A) In blue are represented the biotype 1 isolates, in green the biotype 2 and red the biotype 3. (B) In this case blue are the environmental isolates, in green the clinical and in blue the veterinary strains. Each point represents one strain, and its localization is obtained after applied the two first functions that the discriminant calculated.

Table 21. Some discriminant carbon source for selected groups.

GROUPS	Carbon source (% use)						
	Cellobiose	β-Methyl-D-glucoside	α-D-Lactose	DL-Lactic acid	Glycil-L-aspartic acid	D-Mannitol	DL-α-Glycerolphosphate
Biotyp. 1	+(88.2)	V(37.3)	- (15.7)	+(72.5)	V(58.8)	X	X
Biotyp. 2	+(94.5)	+(83.6)	V(47.3)	V(43.6)	+(85.5)	X	X
Biotyp. 3	- (0)	-(0)	- (0)	+(80)	+(80)	X	X
BT2- SerE	X	X	X	X	X	-(11.1)	V(55.6)
BT2- nonSerE	X	X	X	X	X	+(84.2)	-(26.3)

X. Carbon source without diference in its use between groups
-: ≤30%, V: 30-70%, +:≥70%

Genetic diversity observed with DNA polymorphism loci typing.

The results of the different PCR typing assays are summarized in Tables 17 and 22. The biotype 2 strains were confirmed and subtyped (serovar E versus non-E) using the Biotype 2 Multiplex PCR (Sanjuan and Amaro, 2007). The percentage of each allelic distribution among environmental, human or veterinary *V. vulnificus* isolates was determined (Table 22). In general, the strains could be associated with three profiles: Profile 1 was formed by those strains that were C-genotype, harboured haemolysin type 1 and had 16s rRNA type B. This profile was significantly associated with human septicaemia and seafood isolates of biotype 1. Profile 2 was formed by strains of E-genotype,

type 2-haemolysin and type A-16s-rRNA. This profile was significantly associated with environmental as well as clinical strains mainly of biotype 2. The last profile comprised strains that were E-genotype, type 2 haemolysin and 16S rRNA type AB. The profile 3 was presented only by biotype 3 strains. Approximately 50-80% of biotype 1 isolates from water, fish and wounds showed profile 2. Some exceptions to these main profiles were observed, one of which was the environmental biotype 1 isolate, Riu-1, that gave a mixed profile since it was positive for both types of 16s rRNA as the biotype 3, and presented the haemolysin type 2.

Ribotyping.

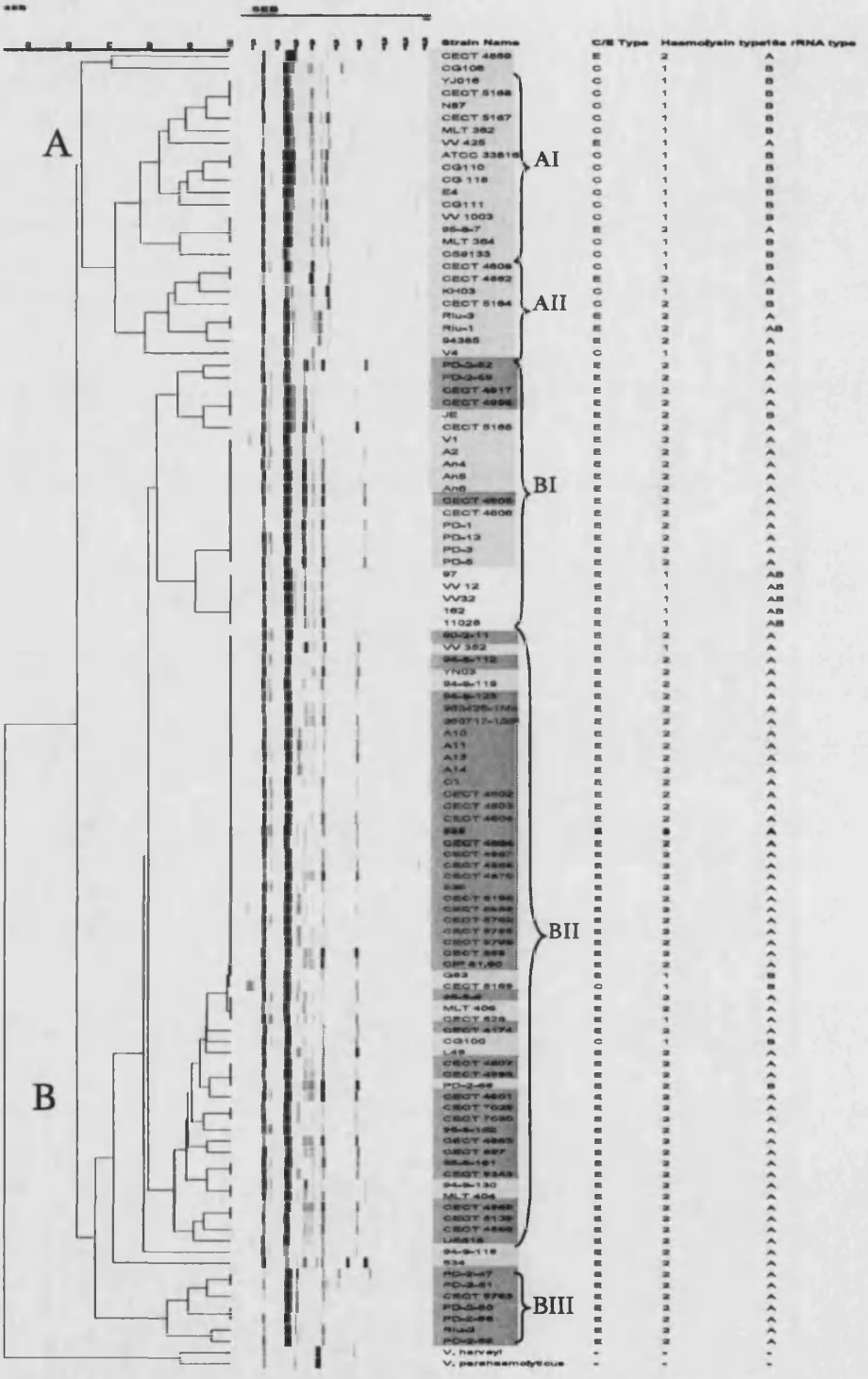
In the ribotyping cluster analysis the bands taken in account were that comprised between 1 to 15 kb. We observed that the riboprinter generated some band of low intensity, especially above 15 kb that probably corresponded to undigested DNA as they were nonreproducible when selected strains were ribotyped twice. The Figure 16 shows the dendogram obtained after UPGMA clustering. Forty seven ribotypes (Rt) were distinguished among the 111 *V. vulnificus* strains (Table 17), which grouped at a similarity of 62%. The other *Vibrio* species included in the study, *V. harveyi* and *V. parahaemolyticus*, showed highly similar profiles (similarity close to 85%) that were clearly different from those of *V. vulnificus*, that grouped at 45% similarity (Figure 16). Two main groups of *V. vulnificus* strains could be distinguished; Division A includes only biotype 1 strains from different sources, most (70 %) of them of profile 1 whereas, Division B included strain of all biotypes, mostly (90%) of profile 2. These two groups could be subdivided into five subgroups (genotypes AI, AII, BI, BII and BIII) based on a similarity ≥80%, even though five strains (CECT 4869, CG106, V4, 94-9-118 and 534) did not cluster to other strains (Figure 16). All the strains of profile 1 were clustered within the genotypes AI and AII with two exceptions, one from a human septicaemia (CECT 5169) and the other an environmental strain (CG100). Clusters AI, AII and BIII included strains of a unique

biotype; cluster AI and AII of biotype 1, mostly of them from humans, and cluster BIII of biotype 2 and serovar E isolated from an unusual source, brackish-water of the estuary of the river Ebro (Mediterranean sea). The rest of the biotype 2 strains were included into the cluster BII, with most exhibiting the same ribotype (Rt 25) while all the strains of biotype 3 were grouped into cluster BI, also showing an unique ribotype (Rt 24).

Table 22. Allelic distribution

Bt	Origin	%											
			Genotype		Haemolysin		16s rRNA			Profile			
		n	C	E	1	2	A	B	AB	1	2	3	At
1	Water	19	21	79	21	79	68.4	26.3	5.3	21.1	68.4	-	10.5
	Seafood	4	75	25	75	25	25	75	-	75	-	-	25
	Environmental	7	42.9	57.1	71.4	28.6	57.1	42.9	-	42.8	28.6	-	28.6
	Fish	5	-	100	20	80	80	20	-	-	80	-	20
	Human blood	12	83.3	16.7	83.3	16.7	16.7	83.3	-	75	8.3	-	16.7
	Human non blood	4	-	100	-	100	50	50	-	-	50	-	50
2	Water	10	-	100	-	100	100	-	-	-	100	-	-
	Healthy fish	2	-	100	-	100	100	-	-	-	100	-	-
	Diseased fish	38	-	100	-	100	100	-	-	-	100	-	-
	Diseased shrimp	1	-	100	-	100	100	-	-	-	100	-	-
	Human blood	2	-	100	-	100	100	-	-	-	100	-	-
	Human non blood	2	-	100	-	100	100	-	-	-	100	-	-
3	Wound	5	-	100	100	-	-	-	100	-	-	100	-

Figure 16. Dendogram derived from a UPGMA cluster analysis of the ribotyping profile. The correspondent allelic results of the polymorphism are also shown.



The strains shadowed in soft grey are biotype 1 and in the dark are the biotype 2 strains. The group of strains without shadow are the biotype 3.

DISCUSSION

In order to analyze the intraespecific diversity of *V. vulnificus,* we have performed a series of phenotypic and genotypic tests with a collection of more than 100 isolates from different biotypes, serovars and sources recovered worldwide. Regarding the phenotypic tests, we used the API 20E and NE systems as well as Biolog tests. The API 20E and API 20NE were previously used unsuccessfully for the identification of clinical and environmental *V. vulnificus* isolates by different authors (Biosca *et al.*, 1993a; Colodner *et al.*, 2004; Dalsgaard *et al.*, 1996a; O'Hara *et al.*, 2003). In this study, we tested 111 strains and found a total of 24 different API 20E profiles, 9 of which had not been published before (Biosca *et al.*, 1993a; Dalsgaard *et al.*, 1996a). The percentage of correct identifications was around 60% regardless biotype, serovar or origin of the isolate with the exception or biotype 3 strains that were correctly identified only in a proportion of 20% Nevertheless, this result is significantly better than those reported by other authors in previous studies, and confirms that the inclusion of the new profiles in the API20E database improves the efficacy of the system in *V. vulnificus* identification. With regard to the differences among the groups of strains tested, the indole test was the only trait with discriminating value. Thus, this trait discriminates biotype 2/ serovar E strains from the rest of the biotypes and serovars since all the serovar E strains tested were negative for this character while other biotypes and serovars were positive. The isolation of some serovar E indole positive strains from environmental samples was previously reported (Amaro *et al.*, 1999), but these strains were further confirmed to belong to biotype 1 by molecular methods (Sanjuan and Amaro, 2007). With regard to API 20NE system, none of the isolates was correctly identified as *V. vulnificus.* We obtained 24 profiles, most of them previously unpublished. No profile could be associated to biotype, serovar or origin, although one profile (5472745) correlated to serovar E since it was exhibited by 73.5% of the trains. Thus, API 20NE should not be use for the

identification of *V. vulnificus* unless the database is amplified with the profiles obtained in this study.

With regard to the Biolog system, results obtained in the present work demonstrate that it was the most effective commercial method for the rapid identification of *V. vulnificus*. In fact, 84% of the isolates were correctly identified, although each isolate presented a unique carbon source use pattern. This result shows the great biochemical heterogeneity of *V. vulnificus*. The number of carbon sources that *V. vulnificus* used ranged from 12 to 53 out of the 95 present o the GN plate with an average of 32. The carbon sources that were species specific following the Alsina's scheme (Alsina and Blanch, 1994a, b) were used by more than 85% of the isolates. One of these carbon sources was the cellobiose that was negative in 80% of the mis-identified strains, including biotype 3 strains that were originally described as being cellobiose negative (Bisharat *et al.*, 1999). This result suggests that this carbohydrate has a great weight for the identification of this species by this system. No specific biochemical profile or test could be associated with any of the tested groups. After discriminant analysis was applied, we were able to establish some guidelines to help in biotyping the isolates, even though the results obtained must be validated with other techniques. Taken together, serovar E and biotype 3 strains were the only groups that could be phenotypically distinguished from the rest on the basis some biochemical characters included in the identification system studied. According to the results obtained by other authors, both groups are quite homogeneous and present some distinguishing phenotypic trait (Biosca *et al.*, 1996b; Bisharat *et al.*, 1999), a fact that facilitates its differentiation.

In order to analyse more deeply the intraspecific diversity of *V. vulnificus*, we also used some genetic methods, as ribotyping and studying the polymorphism at specific loci. Some of these methods have been proposed as genetic tools to distinguish strains of the species with human pathogenic potential (Chatzidaki-Livanis

et al., 2006; Nilsson *et al.*, 2003; Rosche *et al.*, 2005; Senoh *et al.*, 2005).

First, we performed an analysis of three of these putative markers, 16s rRNA, *vvhA*, and C/E typing. According to previous reports, most of *V. vulnificus* human isolates should be C-type, and show *vvhA* type 1 and 16s rRNA type B genotypes, which means that they should exhibit profile 1 of our study. In apparent agreement with these reports, we found an association between profile 1 and biotype 1 human isolates from blood and between profile 2 (E/type- *vvhA* type 2-16s rRNA type A) and environmental isolates, including those from diseased eels, regardless biotyping. However, this association appears spurious since we found that most of the human biotype 1 strains from human wounds and all the strains of biotypes 2 and 3 from humans did not show profile 1, apparently invalidating the previous association. In addition, a high proportion of biotype 1 strains from oysters also showed profile 1. The use in this study of a broad collection of strains that includes clinical and environmental isolates of all biotypes is probably on the basis for this finding. In fact, human and environmental biotype 2 isolates showed profile 2 whereas biotype 3 isolates, all of them from humans, showed profile 3. Thus, the apparent association of profile 1 with *V. vulnificus* isolates with human virulence potential could reflect only a part of the genetic diversity of the species since other human virulent clones are associated with profiles 2 and 3. In conclusion, new genetic markers with epidemiological potential need to be found, since the ones used are not applicable to the species level. Nevertheless, a correlation between profile and biotype was found for biotypes 2 and 3 whereas biotype 1 was more heterogenous including strains of profiles 1 and 2 together with atypical profiles encountered. In case of biotype 2, the homogeneity was extended to all the strains regardless their origins and serovars. These results suggest that this typing scheme could be of epidemiological value for biotype 2 and 3 strains but not for biotype 1 strains.

The genetic characterization of *V. vulnificus* by the ribotyping techniques has been performed in several studies using different enzymes (Amaro *et al.*, 1999; Arias *et al.*, 1998; Arias *et al.*, 1997; Biosca *et al.*, 1997a; Dalsgaard *et al.*, 1996b; Høi *et al.*, 1997). Based on the results of these works we selected *Hind*III to perform the DNA digestion since it has been reported the provided the best banding patterns among enzymes used (Biosca *et al.*, 1997a; Dalsgaard *et al.*, 1996b). In my study, a similar pattern was observed in all of the *V. vulnificus* strains with a group of bands between 2 to 3 kb that were present in all of them but not in the other vibrios included. The ribopatterns were able to cluster *V. vulnificus* isolates in groups with epidemiological significance and in concordance to other typing method used.

The strains that exhibited profile 1 using the PCR polymorphism analysis were located in division A. A second division was formed by all the biotype 2 and 3 strains together with other strains of biotype 1, the latter mainly from environmental sources and wound infections. In this division, the major profile was the second one (E-type, haemolysin 2 and 16s rRNA type A), more related with environmental and eel pathogenic strains. Only two strains (CG100 and CECT 5169) with profile 1 were included in the group BII. Interestingly, the strain CG100 is antigenically related to serovar E strains (Amaro *et al.*, 1999). Group BI was formed by two subgroups, one of which was constituted by biotype 3 isolates, and a second formed by environmental biotype 1 strains related to fishfarms. Biotype 3 is considered to be a clone that recently emerged after a recombination process (Bisharat *et al.*, 2005). Our results suggest that this biotype probably emerged from a biotype 1 clone related to fishfarming.

The eel pathogenic strains were located in the division B with a major ribotype (Rt25) that included strains of the different serovars described as well as others biotype 1 strains mainly from the environment or human wounds. Genogroup BIII is formed by environmental strains isolated from eel tank water and seawater from a sampling site located at the seaside close to the eel farm. The

existence of autochthonous clones of *V. vulnificus* present at the est coast of Spain had been suggested before based on the homogeneous ribotypes that showed those strains compared with other seawater isolates that are more heterogeneous (Arias *et al.*, 1998).

Ribotyping has been used for differentiating clinical and environmental *V. vulnificus* isolates and biotypes (Arias *et al.*, 1998; Arias *et al.*, 1997; Aznar *et al.*, 1993; Tamplin *et al.*, 1996), and several correlations between ribopatterns and geographic origin have been found (Arias *et al.*, 1998; Arias *et al.*, 1997; Tamplin *et al.*, 1996). Our results suggest that this technique could be useful to reveal genetic relationship among the *V. vulnificus* isolates. In this study we were able to assign a specific ribopattern for biotype 3 and for the majority of the biotype 2 strains, regardless of the serovar or isolation origin. Even though we grouped together the human septicaemic strains into the division A, no specific ribopatern could be allocated for them. Furthermore, as we found with the polymorphism study, the rest of the human pathogenic strains were located along the dendogram, invalidating this technique for a rapid identification of the strains with Public Health interest.

To summarize, we have performed an extensive phenotypic and genotypil study in order to analyze the intraspecific diversity of *V. vulnificus*. Our results have demonstrated that the species is heterogeneous at the biochemical level and that no specific profiles could be assigned to a particular group. In fact, the only tests that could differentiate the various groups are the cellobiose test (in the case of the biotype 3) and the indole test (in case of biotype 2 and serovar E). The results of the genotypic study and that of ribotyping suggest that *V. vulnificus* species is subdivided in two main groups, one would include biotype 1 isolates, mainly from human blood and oysters, whereas the other would group biotype 2 and 3 isolates closely related to environmental isolates of biotype 1.

A re-evaluation of the biotypes of the species has been proposed before based on population genetic (Gutacker *et al.*, 2003). In the work of Gutacker *et al.* (2003), the authors found that the species was also subdivided into two major divisions that did not correlate with the biotypes in agreement with our phenotypic and genotypic results. Taking all this in account, a new subdivision scheme that reflects the real population diversity of the species should be developed for *V. vulnificus.*

7

MOLECULAR EPIDEMIOLOGY AND CLONAL COMPOSITION WITHIN *V. vulnificus*

INTRODUCTION

Since present, all phenotypic and genotypic studies performed on *V. vulnificus* indicate that its population structure does not fit with the accepted subdivision into 3 biotypes. For instance, the originally described characteristics for discriminating between biotype 1 and 2 strains lost their value after the isolation of new fish-virulent serovars phenotypically indistinguishable from biotype 1 strains. Further, the results described in the previous chapter demonstrate that *V. vulnificus* is a phenotypically and genotypically diverse species. Thus, practically each isolate gave a different BIOLOG profile and a different ribotype. Nevertheless, statistical analysis of the ribotyping data reveals similarities among the strains, which suggest the strains can be grouped in two genogroups, each showing a main profile by DNA polymorism analysis. These two subdivisions may correspond to those described by Gutacker *et al.* (2003) based on a population genetic study.

Recently, multilocus sequence typing (MLST) was developed to study the phylogenetic and epidemiological relationships among strains belonging to the same or different species (Maiden *et al.*, 1998). This technique is a nucleotide-based approach for bacterial typing. It takes advantage of rapid sequencing technology to reveal allelic variations in internal fragments of some housekeeping genes, with the purpose of characterizing, subtyping, and classifying members of bacterial populations (Urwin and Maiden, 2003). Bisharat *et al.* (2005) developed an MLST scheme that uses the DNA sequences of 10 housekeeping genes for typing and the determining global epidemiology of the newest biotype of *V. vulnificus,* biotype 3. This biotype , since 1996, was responsible for a major outbreak of systemic *V. vulnificus* infections in Israel (Bisharat *et al.*, 1999; Bisharat *et al.*, 2005). These authors applied MLST to a broad collection of strains biased to clinical isolates of biotypes 1 and 3 and found evidence that suggests that biotype 3 is a hybrid

organism that evolved through hybridization of two pre-existing and non-pathogenic populations (Bisharat *et al.*, 2005).

In the present study we have selected four of these housekeeping genes together with three virulence-associated genes to perform a phylogenetic and epidemiological study on our collection of *V. vulnificus* isolates. The objective was to understand the true relationships among the strains and groups, as well as the evolution of this pathogenic species, especially that of the eel pathogenic group.

MATERIAL AND METHODS

Bacterial strains and DNA extraction.

A total of 114 *V. vulnificus* strains of different biotypes, sources and origins were used in this study (Table 23). The genomic DNA of each strain was extracted using standard procedures (Ausubel *et al.*, 1999).

Table 23. Strain used in the MLSA and their allelic result.

Strain	ST[a]	*gip*	*patA*	*pyrC*	*pilA*	*wzz*	*vvhA*	*mdh*	CC[b]	orf 51[c]
YJ106	1	1	1	30	18	1	21	5		-
CECT 4608	2	1	2	12	11	11	2	13		-
BT3-1033	3	2	5	2	10	13	5	10		-
BT3-11028	3	2	5	2	10	13	5	10		-
BT3-vv12	3	2	5	2	10	13	5	10		-
BT3-162	3	2	5	2	10	13	5	10		-
BT3-vv32	3	2	5	2	10	13	5	10		-
BT3-97	3	2	5	2	10	13	5	10		-
A13	4	3	7	3	1	2	7	6	C	+
CECT 5198	5	3	7	3	1	2	7	8	C	+
CECT 7030	5	3	7	3	1	2	7	8	C	+
CECT 7029	5	3	7	3	1	2	7	8	C	+
A10	5	3	7	3	1	2	7	8	C	+
CECT 5343	6	3	7	3	1	2	7	28	C	+
A11	7	3	7	3	1	2	7	32	C	+
CECT 5689	8	3	7	17	1	2	7	29	C	+
CECT 5768	9	3	7	17	1	2	7	30	C	+
CECT 5769	10	3	7	17	1	2	7	31	C	+
L49	11	3	7	26	1	2	7	6	C	-
PD-2-66	12	3	8	26	25	28	15	8		-
JE	13	3	15	13	2	24	1	25		-
CECT 5166	14	3	18	15	14	16	2	14	D	-
CECT 4867	15	4	22	31	26	3	15	33		-

Strain	ST[a]	*gip*	*pntA*	*pyrC*	*pilA*	*wzz*	*vvhA*	*mdh*	CC[b]	orf51[c]
534	16	5	8	4	2	4	12	8		-
CECT 4869	17	5	8	13	2	14	19	1		-
A2	18	6	11	4	2	11	2	7	B	-
PD-1	18	6	11	4	2	11	2	7	B	-
PD-3	18	6	11	4	2	11	2	7	B	-
PD-5	18	6	11	4	2	11	2	7	B	-
V1	18	6	11	4	2	11	2	7	B	-
An4	18	6	11	4	2	11	2	7	B	-
An5	18	6	11	4	2	11	2	7	B	-
An6	18	6	11	4	2	11	2	7	B	-
An7	18	6	11	4	2	11	2	7	B	-
PD-12	19	6	11	4	23	11	2	7	B	-
CECT 4606	20	6	11	4	4	3	2	8		-
MLT 362	21	7	4	21						-
VV 1003	22	8	23	28	27	29	14	24		-
MLT 364	23	9	24	24	8	9	16	8		-
MLT 406	24	10	19	26	2	3	2	1		-
MLT404	25	11	20	25	21	26	7	33		-
ATCC 33816	26	12	9	12	16	12	7	4	E	-
CG100	27	12	9	12	15	20	9	2		-
CG110	29	12	9	19	15	12	4	4	F	-
V 352	30	12	9	29	16	12	17	19		-
CG111	31	12	14	20	15	12	4	2	F	-
CG118	32	12	14	20	15	12	4	4	F	-
CG106	33	12	14	21	17	21	18	3		-
CECT 5167	34	13	14	16	15	17	4	12		-
VV 425	35	13	21	4	18	31	5	27		-
CECT 5164	36	14	3	12	13	15	20	20		-
CECT 5169	37	15	7	4	17	19	11	22		-
CS9133	38	15	14	22	18	22	10	22		-
KH03	39	16	14	12	19	18	4	23	G	-
E4	40	16	14	21	19	18	4	26	G	-
V4	41	17	14	27	18	30	22	23		-
94-9-118	42	18	22	5	2	5	1	8		-
G83	43	19	16	23	20	23	8	8		-
CECT 5168	44	20	7	4	16	18	4	21		-

Strain	ST[a]	*gip*	*putA*	*pyrC*	*pilA*	*wzz*	*vvhA*	*mdh*	CC[b]	orf 51[c]
YN03	45	21	13	9	12	32	7	7		-
94385	46	22	14	8	6	8	8	7		-
536	47	23	10	3	3	2	7	8	H	+
535	48	23	10	3	3	2	7	15	H	+
CECT 5165	49	23	21	14	14	3	2	8		-
Riu-1	50	24	16	8	20	7	8	7		-
94-9-130	51	25	8	7	5	7	1	8		-
Riu-3	52	25	12	3	24	12	1	8	I	-
PD-2-58	53	25	12	3	24	3	1	8	I	+
PD-2-52	54	25	12	3	24	27	1	8	I	+
94-9-119	55	26	17	6	2	6	1	8		-
A14	56	27	7	3	1	2	7	8	C	+
95-8-161	57	28	7	9	7	2	7	16	J	+
95-8-162	58	28	7	9	7	2	7	17	J	+
95-8-6	59	28	7	10	7	2	7	8	J	+
95-8-7	60	28	7	11	8	2	7	8	J	+
CIP 81.90	61	29	7	4	4	3	3	8	A	+
CECT 4862	62	29	8	4	4	3	3	8	A	+
90-2-11	62	29	8	4	4	3	3	8	A	+
94-8-112	62	29	8	4	4	3	3	8	A	+
94-9-123	62	29	8	4	4	3	3	8	A	+
CECT 4174	62	29	8	4	4	3	3	8	A	+
CECT 4601	62	29	8	4	4	3	3	8	A	+
CECT 4602	62	29	8	4	4	3	3	8	A	+
CECT 4603	62	29	8	4	4	3	3	8	A	+
CECT 4605	62	29	8	4	4	3	3	8	A	+
CECT 4607	62	29	8	4	4	3	3	8	A	+
CECT 4863	62	29	8	4	4	3	3	8	A	+
CECT 4865	62	29	8	4	4	3	3	8	A	+
CECT 4866	62	29	8	4	4	3	3	8	A	+
CECT 4870	62	29	8	4	4	3	3	8	A	+
CECT 897	62	29	8	4	4	3	3	8	A	+
CECT 898	62	29	8	4	4	3	3	8	A	+
CECT 4864	62	29	8	4	4	3	3	8	A	+
CECT 4917	62	29	8	4	4	3	3	8	A	+
CCUG 38521	62	29	8	4	4	3	3	8	A	+

Strain	ST[a]	*glp*	*pntA*	*pyrC*	*pilA*	*wzz*	*vvhA*	*mdh*	CC[b]	orf51[c]
PD-2-47	62	29	8	4	4	3	3	8	A	+
PD-2-55	62	29	8	4	4	3	3	8	A	+
PD-2-56	62	29	8	4	4	3	3	8	A	+
Riu-2	62	29	8	4	4	3	3	8	A	+
UE516	62	29	8	4	4	3	3	8	A	+
CECT 4868	63	29	8	4	4	3	3	10	A	+
CECT 4999	64	29	8	4	4	3	3	16	A	+
C1	65	29	8	4	4	3	3	17	A	+
CECT 4604	66	29	8	4	4	3	3	18	A	+
CECT 4998	66	29	8	4	4	3	3	18	A	+
CECT 5139	66	29	8	4	4	3	3	18	A	+
PD-2-50	67	29	8	4	4	3	3	32	A	+
PD-2-51	67	29	8	4	4	3	3	32	A	+
CECT 5762	68	29	8	18	4	3	3	17	A	+
CECT 5763	69	29	8	18	4	3	3	8	A	+
CECT 529[T]	70	29	18	15	14	16	2	14	D	-
960717-1/2F	71	30	6	4	9	6	1	11		+
960426-1/4C	72	30	6	4	9	10	6	11		+
N87	73	31	4	1	22	33	4	9		-

[a]: ST, Sequence type

[b]: CC, clonal complex

[c]: presence of supposed virulence plasmid was checked with orf51 PCR (Chapter 4)

MLSA

Seven loci (table 24), including four housekeeping and three virulence-associated genes, were sequenced. The four housekeeping genes were *glp*, encoding glucose-6-phosphate isomerase; *mdh*, encoding malate-lactate dehydrogenase; *pntA*, encoding the transhydrogenase alpha subunit; and *pyrC*, encoding dihydroorotase. These loci are a subset of the original *V. vulnificus* MLST scheme (Bisharat *et al.*, 2005) and were chosen because they provide robust data and, along with the virulence genes examined (see below), could resolve evolutionary relationships. Primers, PCR conditions, and sequences of these loci were described previously by Bisharat *et al.* 2005).

The virulence-associated genes were ***pilF***, encoding a type IV fimbrial assembly protein; ***vvhA***, encoding the haemolysin/ /cytolysin; and ***wwz***, encoding the O-antigen chain length regulator. The same primers were used for PCR amplification and sequencing and were designed using Primer3 software (Rozen and Skaletsky, 2000) using the consensus sequences obtained after aligning the sequences of the loci of the two published *V. vulnificus* genomes (Chen *et al.*, 2003; Kim *et al.*, 2003b).

Table 24. Primers and PCR conditions used in the MLSA

Locus	Sequence primers
glp	AGTCGATCCTGAGACAACACTC GCCTTGGTGGATCAGTTGGTA
mdh	ACTAGAAGGTGCTGATGTGGTT TGTGCAAAGAAGCTAGCATGCT
pntA	GTTATCGAGCGGTTGTTGAAG CCGATCACTTTTACGCCGTT
pyrC	GATCGCATTCAAGCACATAACC GTCCGTACCCAAAAAGAACTTC
vvhA	GATGTTCGCGTCAATGTGG GTGCTTCCGCTTCAAACAG
pilF	GCCGCAATTGGTAGGCAC GCAGCCCATAGATTTGGT
wwz	AAGCAGTTCCGCACCTTG TTGCCACAACGGATTCCT

In all cases the PCRs were performed in 50 µl-reaction volumes that contained 1X High Fidelity Expand PCR system buffer (Roche Diagnostics), 1.5 mM $MgCl_2$, 200 µmol of each deoxynucleoside triphosphate (Roche), 1 U of High Fidelity Expand PCR system polymerase (Roche Diagnostics), and 100 pmol of each primer. The reaction conditions were an initial denaturation step at 94ºC for 4 min, followed by 30 cycles of 1 min at 94ºC for

denaturing, 45 s at 50ºC for annealing, and 1 min at 72ºC for extension, with a final extension step at 72ºC for 10 min. Sequencing was conducted with an ABI3700 automated sequencer using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's specifications.

Sequences for each locus were aligned using Vector NTI 9.0.0 software (Infomax) and were edited manually by eye. Different allelic sequences within a locus were assigned arbitrary numbers. Each isolate was consequently given a seven number sequence that represented its different allelic sequences and this coded results is known as sequence type (ST), and each ST was numbered in order of appearance (ST-1, ST-2, and so on).

In order to test the consequence of natural selection on the loci studied, the number of polymorphic nucleotide sites and the ratio of the number of non-synonymous to the number of synonymous substitutions (the d_N/d_S ratio) were calculated using the START program (Jolley *et al.*, 2001) (Table 25). Allele sequences for each strain were concatenated and used to construct a dendogram using the neighbour-joining method and the Kimura 2-parameter distance estimation method. Reliability of the tree structure was assessed via bootstrap analysis with 1000 replicates. Phylogenetic analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004).

RESULTS

Genetic diversity observed by MLSA.

We identified 73 different sequence types (ST) among the 115 strains analyzed, with ST-62 being the most frequently observed (almost 21% of the isolates). The number of alleles found at each gene ranged from 22 in *vvhA* to 33 in *wwz* (table 25). The ratio of nonsynonymous to synonymous nucleotide changes within a gene, d_N/d_S, is a measure of the history of selection for change in that gene. High d_N/d_S values indicate there has been selection pressure for novel forms, while low d_N/d_S values mean selection has been acting to conserve the sequence. In our cases, the d_N/d_S ratio was always lower than 0.1, indicating that the *V. vulnificus* MLSA loci were not subject to positive selection. The 32 STs identified could be organized into 11 clonal complexes (CC), and these were composed of strains with either identical STs, or STs that varied at one or two loci. Two of these groups contained a predicted founder ST (an ST to which the other STs in the CC were related). The strains included in each of the CC are indicated in Table 23 and eBURST diagram of the population of *V. vulnificus* is shown in Figure 17.

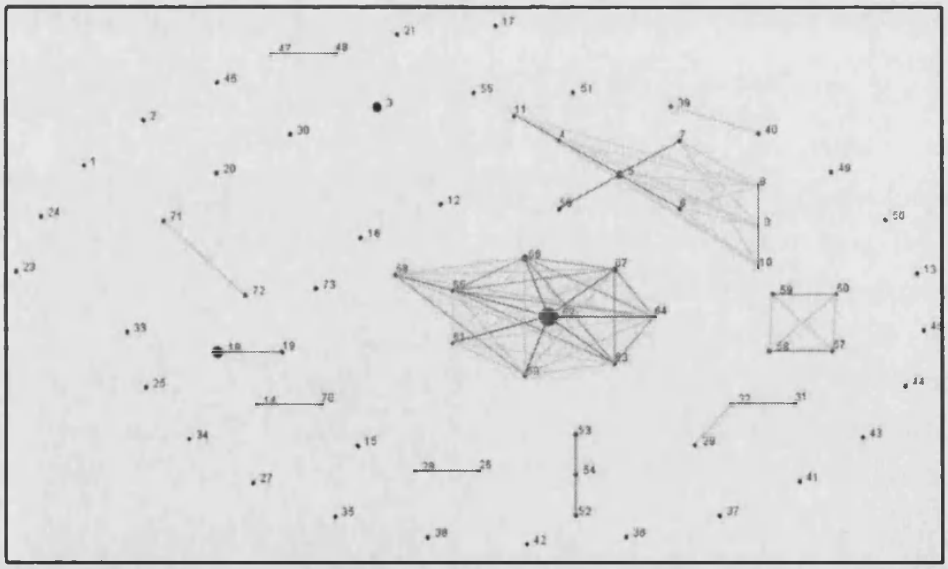
Different phylogenetic trees were constructed using the housekeeping or the virulence-associated gene sequences. Finally, an NJ tree was constructed from concatenated sequences of the seven loci. Overall, clustering generated from composite sequences was congruent with the CC formed with the MLSA allelic profiles. The dendogram obtained with the concatenated sequence of the housekeeping genes could be rooted using *V. parahaemolyticus* (accession number BA000031 and BA00032) and *V. cholerae* (AE003852 and AE003853) (Heidelberg *et al.*, 2000; Makino *et al.*, 2003) as outgroup. In all trees, the strains were divided into two major divisions, and in general, the same strains were always located in the same group. Division I included strains of all three

biotypes which were isolated from different environmental and clinical (human or animal) sources. In contrast, division II included only biotype 1 strains, most of which were obtained from human septicaemias (Figure 17 and Table 23).

Table 25. Allelic polymorphisms in the seven genes fragment anslyzed.

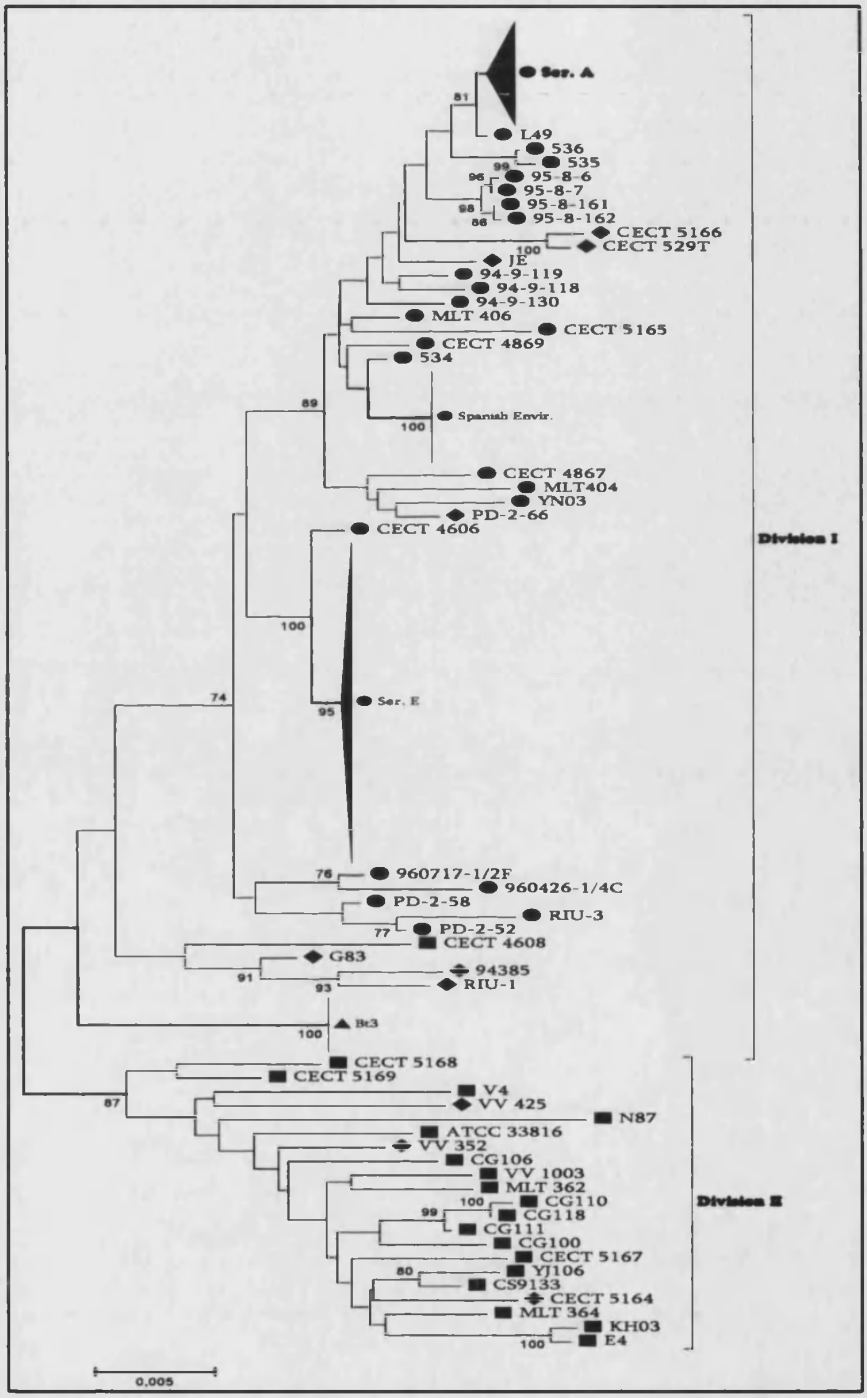
Locus	Fragment size (bp)	№ of alleles	№ of variable sites	% variable sites	dN/dS
mdh	459	32	44	9.6	0.0033
pyrC	423	31	39	9.2	0.0142
pntA	396	24	34	8.6	0.0086
glp	480	31	50	10.4	0,0142
pilF	484	27	57	11.8	0.0393
vvhA	432	22	32	7.4	0.0021
wwz	462	33	61	13.2	0.0583

Figure 17. eBURST diagram of the *V. vulnificus* population.



Numbers correspond to the STs (Table 23). Blue circles indicate a predicted founder and black lines link ST of the clonal complex. Pink lines link STs that varied in one locus and blue lines those that varied in two loci

Figure 18.NJ dendogram derived from concatenated sequence of the 7 loci studied.



Profiles obtained in the polymorphism study (chapter 6): ■ profile 1, ● profile 2, ▲ profile 3, ♦ atypical profile.

Figure 19. NJ dendograms derived from the different allelic sequence of virulence associated genes studied.

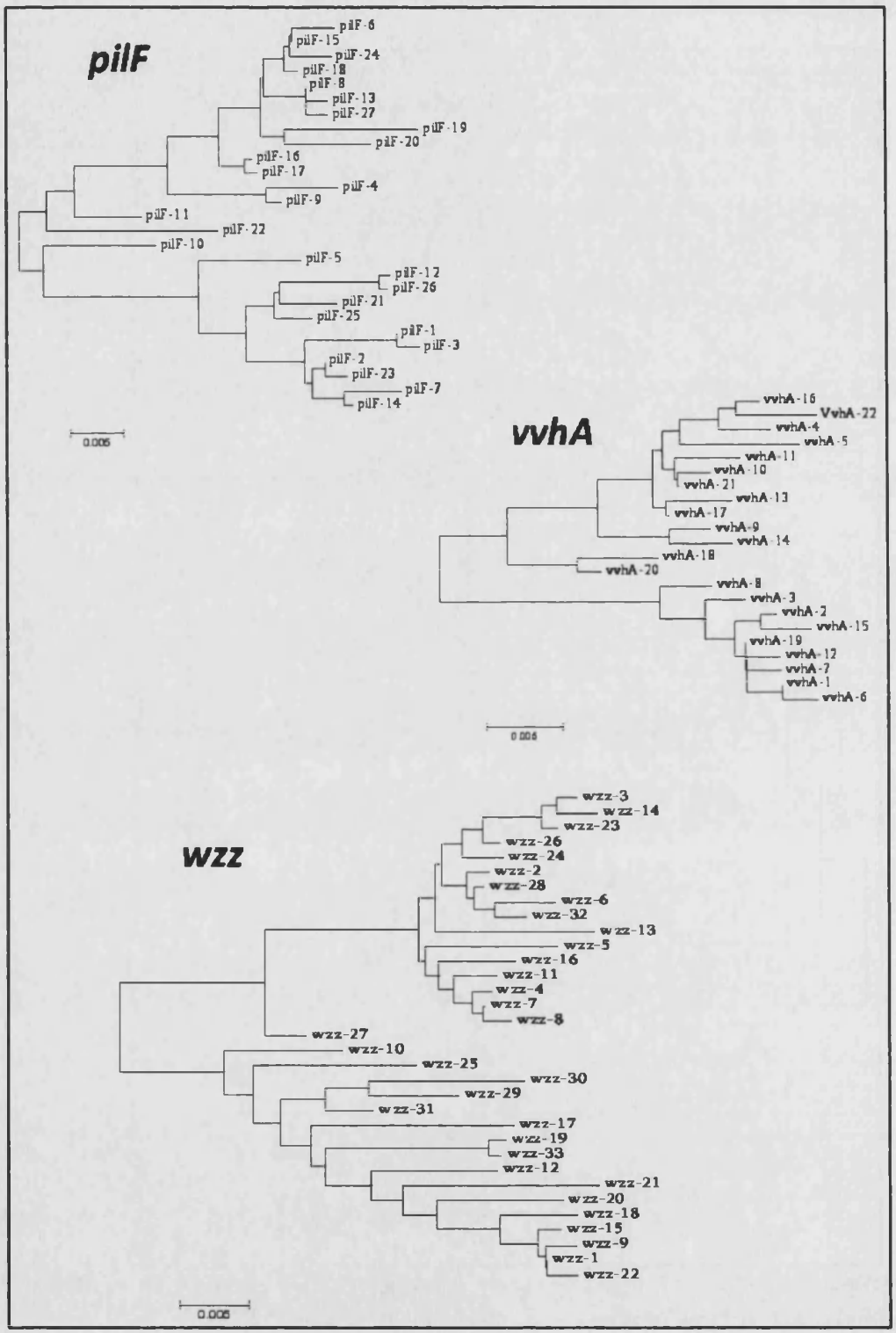


Figure 20. NJ dendograms derived from the different allelic sequences of housekeeping genes studied

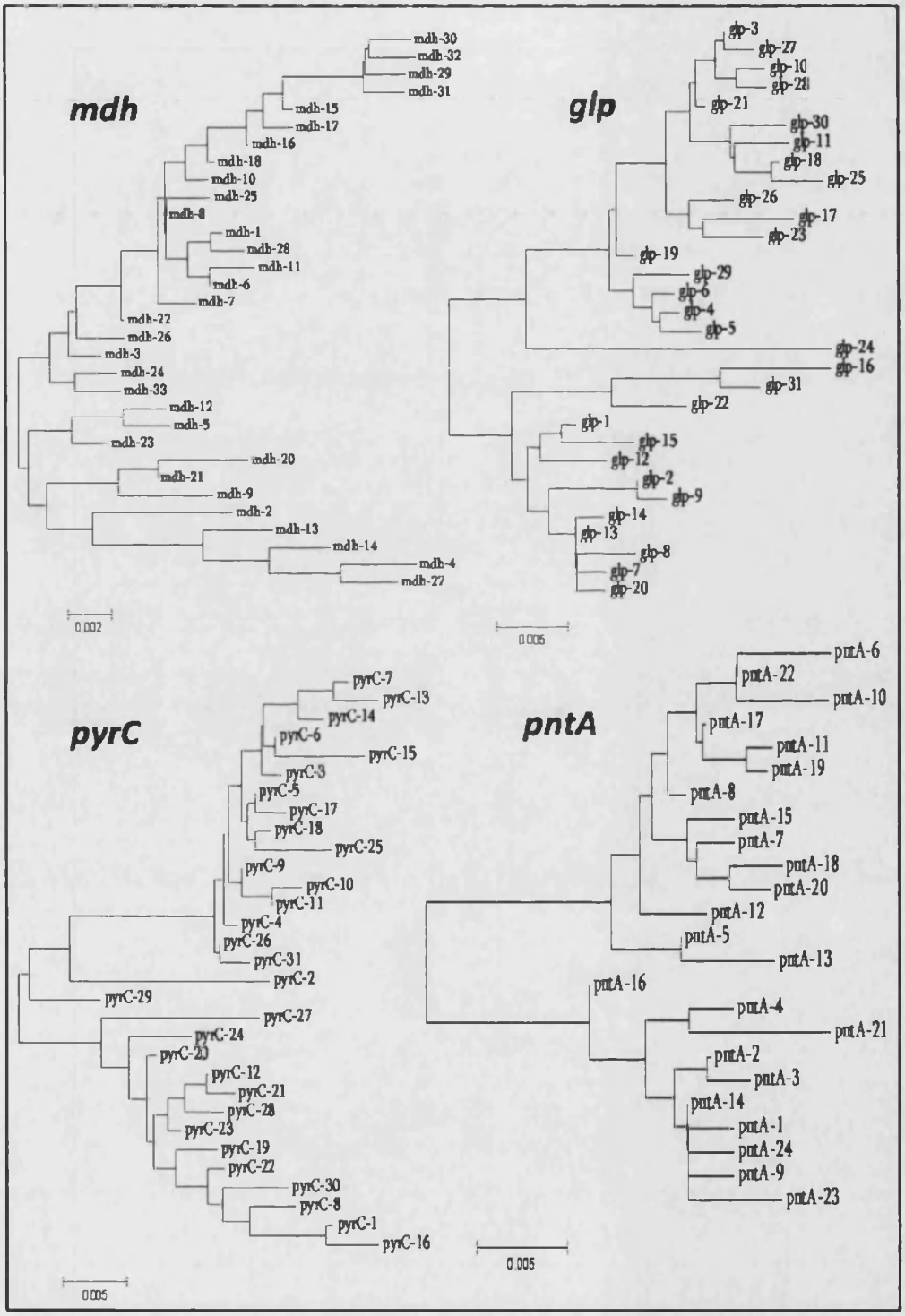
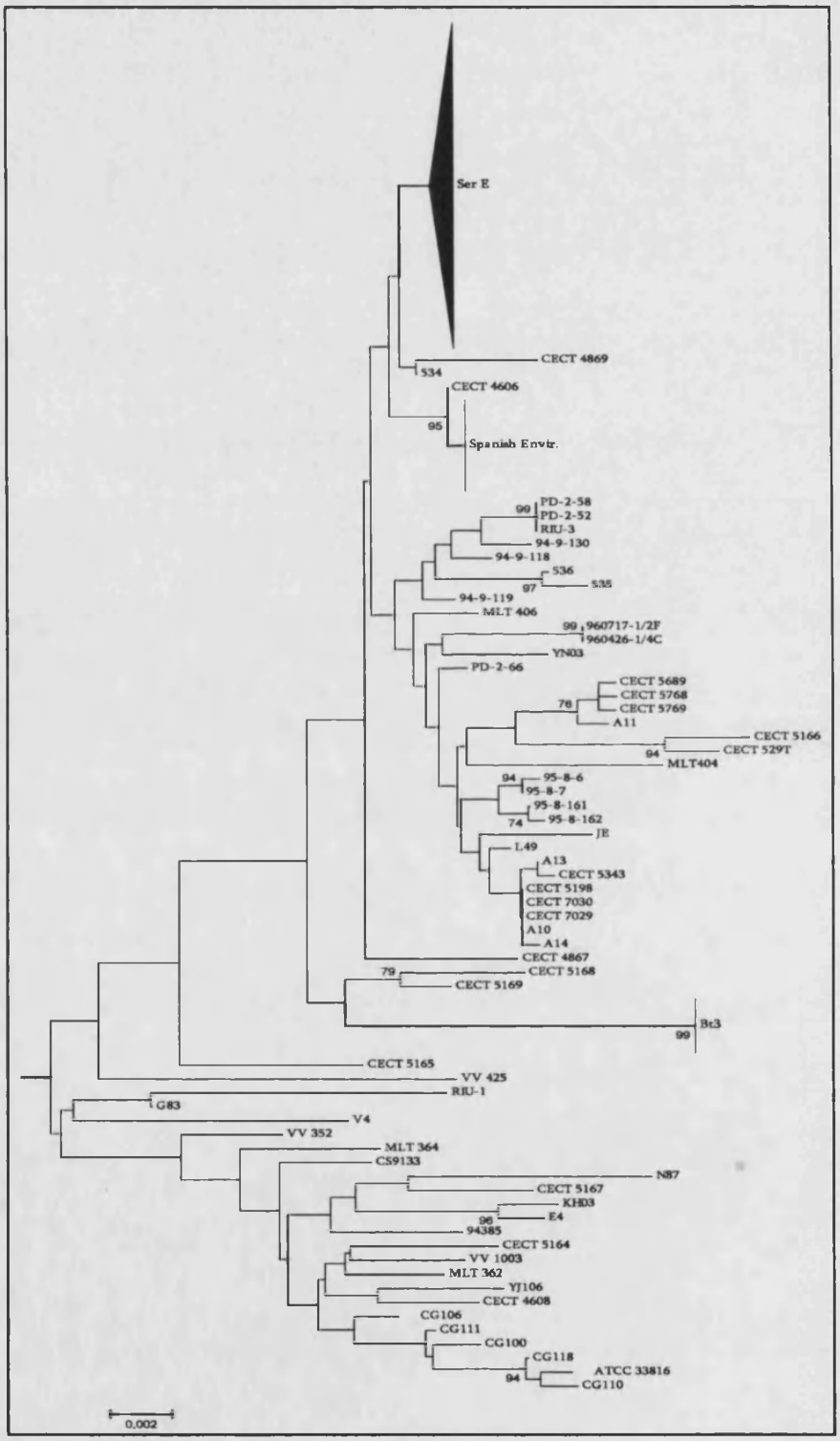


Figure 21. Rooted NJ dendograms derived from sequence of the 4 housekeeping loci studied



DISCUSSION

In a previous study, using the phylogenies generated by multilocus enzyme electrophoresis (MLEE), *recA* and *glnA* gene sequence and RAPD analysis, Gutaker *et al* found evidence for the existence of a distinct genetic subgroup associated with eel virulence. These authors found that indole-negative strains, which correspond to serovar E, tended to cluster together although the eel-virulent strains that were indole positive had different variable positions among the phylogenies constructed (Gutacker *et al.*, 2003).

Our study has also revealed genetic diversity among the biotype 2 strains and the MLSA and phylogenetic results support the hypothesis that biotype 2 of *V. vulnificus* may have emerged from multiple, independent origins. For instance, in all trees, the different serovars of this biotype formed defined and separate clusters, although it seems that the serovar A and serovar I strains are more closely related. Similarly, the sequences of the studied loci in the different serovars of biotype 2 are different, although the serovar A and I strains shared the same sequence in two loci (*pntA* and *vvhA*). Those serovars may share a common ancestor that could have acquired the virulence plasmid, although a study of the divergence of the plasmid sequence is necessary to confirm this hypothesis. The polyphyletic origin of the biotype is evident from an examination of the different trees constructed. In fact, with the exception of the strains of biotype 3, no correlations were observed between biotypes and the phylogenies generated by the sequences from this study, or with those previously generated by other authors (Bisharat *et al.*, 2005; Gutacker *et al.*, 2003).

In all the dendrograms derived from either monolocus or concatenated sequences, strains were divided into two divisions that included the same strains. This general topology included two exceptions. First, while the *pilF* locus also exhibited a division into two groups, the strains that formed the groups were different; biotype 3 or serovar E strains clustered together with other biotype

1 strains compared with other trees. The other discrepancy is the *mdh* locus that presents a completely different topology, with some strains that clustered together in other loci having phylogenetic differences in the *mdh* loci.

The distribution of the *V. vulnificus* population into two large divisions was previously observed using different methods (Bisharat *et al.*, 2005; Chatzidaki-Livanis *et al.*, 2006; Gutacker *et al.*, 2003; Nilsson *et al.*, 2003; Rosche *et al.*, 2005; Senoh *et al.*, 2005; Warner and Oliver, 1999). The tree generated in the present study employing the studied locus sequences also generated two divisions (I and II), which are similar to those observed in the phylogenies obtained by Gutaker *et al.* (Gutacker *et al.*, 2003). We observed that the profiles described using DNA polymorphism typing could be associated with one of those divisions, so that division I included strains with profiles 2 and 3, while division II was associated with profile 1. We also observed this division in the dendogram derived of the riboprinting study. In that case, in general, the same isolates for the equivalent divisions and even a few clusters were identical by both techniques (e.g. biotype 3 or the Spanish isolates), although the rest of the groups and positions among the dendogram differed considerably between them.

Despite the fact that all *V. vulnificus* strains must be considered as representing possible human pathogens, it seems that the strains of division II may share some determinants that make them more virulent since most of the septicaemic strains fell into this division. Moreover, in the *pilF* tree, a rearrangement of the strains was observed and, with few exceptions, all the human isolates clustered together, including those of serovar E whose human potential virulence has been previously demonstrated (Amaro and Biosca, 1996; Sanjuan and Amaro, 2004). This result is quite interesting in that it suggests that a genetic grouping of *V. vulnificus* strains on the basis of a partial sequence of this gene may be a suitable system for classification of the strains according to their human virulence potential. In addition, a PCR method using primers from the selected regions of this gene could be developed

and validated for a rapid detection in seafood of those strains that pose a risk for public health. The importance of the type IV pili (Tfp) in bacterial virulence has been demonstrated in other studies. For example, in *V. vulnificus* biotype 1, a mutation of the *pilA* (the pilin) gene decreases adherence to Hep-2 cells and associated virulence in iron dextran-treated mice (Paranjpye and Strom, 2005). In the case of PilF, which is a protein required to assemble pilin into Tfp, no pili were detected when this protein was mutated in *Neisseria meningitidis* (Carbonnelle *et al.*, 2006).

Analysis of the MLSA data has confirmed that *V. vulnificus* has a non-clonal population structure, as 67 of the 73 STs found correspond to a single isolate. This suggests a high genotypic variation of the species. However, some clones or clonal complexes were detected. For example, those formed by several environmental strains isolated from Spain (CC-B) or the different serovars of biotype 2: serovar E (CC-A), serovar A (CC-C) and serovar I (CC-J). Other minor clonal complexes were formed by two or three isolates taken from the same geographical and even temporal sources. The biotype 3 strains included in this study represent a unique ST (ST-3), which is in agreement with an MLST assay previously performed where 62 biotype 3 strains were found to be genetically identical in the 10 housekeeping genes studied (Bisharat *et al.*, 2005). The lack of genetic diversity in biotype 3 strains suggests that these strains form a clone that has evolved recently, as was proposed previously (Bisharat *et al.*, 2005).

The descriptions of the biotype 2 strains by Tison et al (1982), and later the biotype 3 strains by Bisharat *et al.* (1999) were based on phenotypic results of some strains that belong to two clones isolated from an epidemic process, in Japanese eels and in humans respectively. Their special virulent properties lead to an increase in the number of isolates in those clones with respect to the other environmental strains. With this biased collection the definition of biotypes could be satisfactory but, when the complete *V. vulnificus* population is studied, the division is no longer suitable.

In conclusion, the actual sub-classification of *V. vulnificus* species into biotypes is supported neither by our phylogenetic data nor the polymorphisms in the different loci examined. In the case of the so-called biotype 2 strains, the data confirm that they have emerged on different occasions, probably by the acquisition of a virulence plasmid. This polyphyletic origin supports its reclassification of these strains into a pathovar within the species. This pathovar would include the strains of this species with pathogenic potential to infect and develop vibriosis in fish. The diversity among the biotype 1 strains alone confirms that they must be classified using other criteria. The data show that there are two distinct *V. vulnificus* populations that are correlated with the distinct genotypes that could be used for the reclassification of the species.

8

CONCLUSIONS

From the results obtained throughout this study, the following main conclusions have been reached:

1. ***V. vulnificus* biotype 2 serovar E is an opportunistic pathogen for humans and fish whose main reservoir is the aquatic environment.** The new two-step protocol together with the multiplex PCR designed in the present work have been successfully applied to environmental samples allowing the isolation of new serovar E strains from sea water and healthy fish, which demonstrates that this serovar is naturally present in the aquatic environment in absence of eels and epizootics. In addition, the application of the multiplex PCR to a wide collection of *V. vulnificus* strains has allowed the identification of new serovar E isolates from humans, which indicates that the importance of this serovar as human pathogen has probably been underestimated.

2. **A specific protocol for the isolation of serovar E strains from environmental samples has been designed.** The key element of this protocol is the enrichment broth used in the first step, SEB-1, which contains fresh eel serum as selective component. SEB-1 yields differences in bacterial counts between serovar E and competitors (including biotypes 1 and 3) high enough to allow the isolation of this serovar from natural, mixed populations after only 8 h of incubation. SEB-1 also could be applied for the selective isolation of the rest of serovars of biotype 2 since they also resist the bactericidal action of fresh eel serum. For the second step, the selected selective and differential agar, VVM agar, gives the highest plating efficiency compared to those recommended by the FDA, CPC and derivatives, not only for the serovar E group but also for the rest of *V. vulnificus* groups, including biotype 3.

3. **The environmental strains of *V. vulnificus* biotype 2 and serovar E possess the virulence factors essential to develop human and fish vibriosis.** The environmental strains of this serovar resist the bactericidal action of eel and human serum and are as virulent for mice and fish as the clinical isolates, which suggest that the

origin of the isolate does not predict its virulent potential in the biotype 2 of *V. vulnificus* species.

4. **The genomes of the biotype 1 and biotype 2 present high homology.** The low number of specific sequences found after comparison of the genomes of fish virulent and avirulent *V. vulnificus* strains by SSH suggests that biotype 1 and biotype 2 strains share a high proportion of genetic information in their genomes, in spite of its phenotypic and genotypic differences.

5. **The biotype 2 strains possess specific DNA sequences located on plasmids that could be related to fish virulence and have been acquired by horizontal transfer.** We have identified six DNA sequences by SSH, three plasmidic ones, specific for biotype 2, and three chromosomal ones, specific for the zoonotic serovar.

6. **A multiplex PCR assay for the rapid identification of *V. vulnificus* biotype 2 that allows the discrimination of the zoonotic serovar has been developed and validated in the present work.** The protocol uses three primer pairs, one specific for the species and the other two specific for biotype 2 and serovar E, respectively. The biotype 2 and serovar E-specific primers were designed from the DNA sequences obtained by SSH .The PCR could be applied for both the rapid diagnosis of fish and human vibriosis and, combined with a previous enrichment in SEB-1, for the specific detection of healthy carriers destined to human consumers. In this case, the designed sampling procedure from surface mucus could be performed without killing the animals. In addition, the multiplex assay could be used in epidemiological studies to correctly biotype clinical isolates and clarify the status of serovar E as human pathogen.

7. **The species *V. vulnificus* is phenotypically heterogeneous as it was revealed by the phenotypic assays performed in the present study.** No API20E, API20NE and BIOLOG profile could be associated to biotype, serovar or origin, although all serovar E

strains were indole negative in API20E and presented the API20NE profile 5472745 with a probability of 73.5%, and all the biotype 3 strains could be clearly differentiated from the rest of strains on the basis of the utilization of cellobiose as unique carbon source. Taken together, the serovar E and the biotype 3 strains were the only groups that could be phenotypically distinguished from the rest on the basis of particular results in some of the biochemical characters included in the identification system used.

8. **From the commercial systems; API20E, API20NE and BIOLOG, the more adequate one for species identification is the BIOLOG system, whereas none of the systems provides a correct identification at subspecies level**. Currently, the API20E system has improved its accuracy for *V. vulnificus* identification up to 60%. With the inclusion of the new profiles found in the present work its effectiveness could increase and achieve acceptable values. In this case, this system should be preferentially used because is the cheapest and easiest to interpret and, at the same time, it allows a presumptive identification of the zoonotic serovar based on the result in indole test.

9. ***V. vulnificus* biotype 1 is genotypically more heterogeneous than biotype 2 and biotype 3**. Three main genotypes were found among *V. vulnificus* isolates; profile 1, mostly found among oyster and blood human isolates of biotype 1, profile 2, among environmental biotype 1 isolates and biotype 2 isolates regardless the type of sample and the serovar, and profile 3, among biotype 3 isolates. After ribotyping, the *V. vulnificus* isolates were subdivided in two main divisions, division A groups mainly biotype 1 isolates from human blood and oysters whereas division B groups biotype 2 and 3 isolates closely related to environmental isolates of biotype 1 mainly from fishfarm environments. Although a main ribotype was presented by biotype 2 isolates, this group is more heterogenous than the group formed by biotype 3 isolates, which only presented one ribopattern.

10. ***V. vulnificus* species is subdivided in two main divisions, one that gave rise to the biotype 2, 3 and part of biotype 1and the other one that gave rise to a heterogeneous conjunct of biotype 1 strains mainly from human origin**. This conclusion was obtained after sequencing 4 housekeeping and three virulence genes and further analysis of the MLSA data, and confirms the results obtained in the pheno- and genotypic characterization. These two divisions (*environmental* and *clinical*) are highly correlated with the ones found by ribotyping.

11. ***V. vulnificus* species has a non-clonal population structure**, although some punctual clones or clonal complexes, mainly those formed by the biotype 2-serovar E, biotype 2-serovar A-I and biotype-3 strains, have been formed. The outbreaks in animals or humans lead to increase the number of isolates of those clones.

12. **Biotype 2 strains have a polyphyletic origin.** The phylogenies obtained in this study have revealed that in the biotype 2, serovar A is closer to serovar I than to serovar E, which is closer to biotype 1 isolates from fish farm environments. This result suggests that biotype 2 isolates could have emerged from different clones of the *environmental division* by independent acquisition of the virulence plasmid.

13. **The actual subclassification of the *V. vulnificus* species into biotypes is supported neither by our phylogenetic data nor the polymorphisms on the different loci examined.**The polyphyletic origin of the biotype 2 strains supports its reclassification as a pathovar within the species. This pathovar would group the strains of the species with pathogenic potential to infect and develop vibriosis in fish. The diversity among the biotype 1 strains confirms by itself that they must be classified using other criteria. The data show that there are two distinct *V. vulnificus* populations that are correlated with the distinct genotypes that could be used for the reclassification of the species. In addition, a genetic grouping of *V. vulnificus* strains based on the partial sequence of *pilF* may be a suitable system for classification of the strains according to their

human virulence potential, since with this system human virulent strains clustered together irrespective to their biotype or serotype.

9

RESUMEN
SPANISH SUMMARY

PERSPECTIVA GLOBAL DEL GÉNERO VIBRIO

El género *Vibrio* comprende un gran número de especies de γ-proteobacterias Gram-negativas de forma bacilar, recta o curvada y móviles mediante flagelos polares. Los vibrios son bacterias anaerobias facultativas, quimioorganotrofas y halófilas, con la excepción de *Vibrio cholerae* y *V. mimicus* que no presentan requisitos de Na^+. Los vibrios producen una gran variedad de enzimas que degradan macromoléculas, como proteasas, lipasas y quitinasas.

Son bacterias acuáticas que se aíslan por todo el planeta y cuya distribución depende del contenido en sodio y nutrientes del hábitat así como de la temperatura. Representan el grupo mayoritario de bacterias cultivables en ambientes marinos y estuarinos. Muchos estudios han revelado que también son abundantes en o sobre organismos marinos; aislándose de la superficie de corales, peces, moluscos, algas, esponjas, gambas o zooplancton, así como del intestino de algunos de estos organismos.

Entre los vibrios y los organismos pluricelulares acuáticos pueden establecerse relaciones parasíticas, simbióticas y comensales, de las cuales la más estudiada es la primera dada la capacidad que tienen algunas especies de producir enfermedades en una gran variedad de hospedadores (ver Table 1).

RELACIONES PARASÍTICAS: PATOGENECIDAD

Humanos

Hasta la fecha se han descrito 12 especies de vibrios patógenas para el hombre, siendo *V. cholerae*, *V. parahaemolyticus* y *V. vulnificus* las más importantes tanto en número como en gravedad de los casos. Las patologías humanas pueden clasificarse por su sintomatología en tres tipos: Gastroenteritis, septicemia primaria e infección de heridas.

Otros organismos

Los vibrios pueden causar enfermedades en animales que co-existen con ellos en su hábitat natural o en ambientes artificiales tales como las granjas de cultivo de peces y mariscos. Estas infecciones se suelen denominar vibriosis.

- Vibriosis de los peces. Es una de las enfermedades más frecuentes en peces en cultivo, afectando generalmente a especies marinas y de aguas salobres. La vibriosis de los peces es una enfermedad septicémica con alta tasa de morbilidad y mortalidad, siendo la de mayor distribución y frecuencia la causada por *V. anguillarum*.
- Vibriosis luminosa (de los peneidos). Recibe este nombre porque en paralelo al proceso infeccioso, los animales se vuelven luminiscentes debido al crecimiento del agente causal, *V. harveyi* que es un vibrio luminiscente. Es una enfermedad septicémica que afecta sobre todo en estado larvario y que provoca un menor crecimiento de los animales junto con una tasa variable de mortalidad.
- Vibriosis de los bivalvos. Como en los peces, es la infección por vibrios la más abundante en criaderos de moluscos. Es una enfermedad sistémica de los tejidos blandos que suele acabar con la muerte debido a necrosis tisular.
- Blanqueamiento de los corales. A finales de la década de los 90 se demostró aplicando los postulados de Koch que *V. shiloi* era el agente etiológico de una enfermedad que es una de las causas de la destrucción de los arrecifes coralinos de *Oculina patagonica* cuyo síntoma es el blanqueamiento del coral por la pérdida del alga simbionte.

RELACIONES SIMBIÓTICAS

Entre las relaciones simbióticas de *V. spp* con otros organismos, la más estudiada es la simbiosis entre *V. fischeri* y el calamar nocturno *Euprymna scolopes*. La bacteria se instala en el órgano luminoso y produce bioluminiscencia que ayuda al calamar a camuflarse de posibles depredadores. Durante la noche, cuando sale el calamar, éste expulsa la mayoría de las bacterias al agua y durante el día, cuando se esconde debajo de la arena, los vibrios que han quedado se multiplican de forma que a la llegada de la noche vuelve a estar el órgano lleno de bacterias y vuelve a emitir luz. Esta interacción se considera un modelo para el estudio de las relaciones simbióticas dado que ambos organismos son fácilmente cultivables en laboratorio.

VIBRIO VULNIFICUS

CONTEXTO HISTORICO

En 1976, se describió un vibrio halófilo, fenotípicamente similar a *V. alginolyticus* y *V. parahaemolyticus,* que también podía causar enfermedad en humanos, pero que a diferencia de los anteriores podía fermentar la lactosa, por lo que se le denominó "vibrio lactosa positivo". Más tarde se incluyó en el género *Beneckea* con el nombre de *Beneckea vulnifica* y posteriormente se reasignó al género *Vibrio* con su nombre actual, *Vibrio vulnificus.*

También en 1976 Muroga y Nishibuchi aislaron de anguila japonesa enferma unas bacterias que en 1982 Tison et al, compararon con aislados ambientales y humanos de *V. vulnificus* concluyendo que los aislados de anguila pertenecían a la misma especie aunque presentaban algunas peculiaridades fenotípicas. En base a estas peculiaridades, los autores propusieron la división de la especie en dos biotipos; el biotipo 1 incluía los aislados "típicos" de origen humano y ambiental, y el biotipo 2 incluía los aislados

"atípicos" de patógenos de peces. En el año 1999, se describió un tercer biotipo que incluye cepas aisladas en Israel de pacientes con infecciones graves en heridas tras el contacto con tilapias cultivadas. Estas cepas presentan también unas peculiaridades bioquímicas y genéticas que justificarían la creación de un tercer biotipo.

SUBDIVISION DE LA ESPECIE

V. vulnificus es una especie heterogénea que ha sido subdividida en biotipos, serotipos y genotipos, aunque ninguna de esas clasificaciones sirve para diferenciar claramente las cepas virulentas para el hombre de las que no lo son.

Subdivisión serológica

La especificidad antigénica se ha usado para la identificación de la especie y la variabilidad antigénica para su subdivisión. Así, para la identificación de la especie puede utilizarse la aglutinación con anticuerpos frente al antígeno flagelar (antígeno H) y para la subdivisión de la especie distintas técnicas serológicas basadas en el lipopolisacárido (LPS) o antígeno O. En la actualidad no existe ninguna clasificación serológica subespecífica que se utilice en todo el mundo. Se han propuesto 3 diferentes:

1. Sistema de Shimada y Sakazaki; basado en la aglutinación de células enteras con anticuerpos policlonales de conejo frente a células inactivadas por calor. Este sistema reconoce al menos 7 subgrupos diferentes pero tiene el inconveniente de que hay mucha reacción cruzada entre los distintos grupos serológicos.
2. Sistema de Martin y Siebeling; basado en la técnica ELISA con anticuerpos monoclonales obtenidos frente al LPS purificado. El sistema no está del todo desarrollado ya que los 5 serotipos descritos no son reflejan la diversidad serológica de la especie.

3. Sistema de Biosca *et al.* Este sistema se desarrolló para el biotipo 2 de la especie y ha sido usado con éxito en con el biotipo 3 y con parte del biotipo 1. Se basa en una tinción inmunológica del LPS con anticuerpos policlonales obtenidos en conejo inmunizado con células enteras inactivadas con formol. Con este sistema se ha subdividido el biotipo 2 en tres serovares (E, A e I) y se ha constatado la especificidad y homogeneidad serológica del biotipo 3 así como la variabilidad del 1. La serovariedad E del biotipo 2 es la más virulenta para la anguila y la única asociada a vibriosis humanas. A esta serovariedad pertenecen los aislados que uso Tison *et al.* para la descripción original del biotipo 2.

Biotipos

Ya hemos indicado que actualmente la especie se subdivide en tres biotipos con características bioquímicas así como rango de hospedadores y propiedades epidemiológicas *a priori* diferenciales (ver Table 4). Todos los biotipos pueden ser patógenos para humanos, pero sólo el biotipo 2 incluye cepas patógenas para peces. En la actualidad, las características diferenciales no son válidas, ya que hay aislados de peces enfermos que no pueden adscribirse al biotipo 2 porque de acuerdo con los criterios originales pertenecerían al biotipo 1. En estos momentos, sólo el biotipo 3 se comporta como un grupo homogéneo y con características similares y diferentes al resto de los aislados de la especie, lo que pone en entredicho la actual división ya que no refleja las verdaderas relaciones entre los aislados.

Aislados clínicos *Vs* ambientales

Dado el potencial virulento para el hombre de esta especie, son varios los estudios que han intentado encontrar algún marcador

genotípico o fenotípico que permitiera diferenciar las cepas con poder virulento para el hombre (*clínicas*) de las que no lo tienen (*ambientales*). Algunos de estos trabajos proponen posibles marcadores en base a estudios realizados con cepas de biotipo 1. Un ejemplo es el estudio realizado por Aznar *et al* que encuentra dos variantes de la secuencia del gen 16s del ARNr; la A presente en aislados ambientales y la B en aislados clínicos.

ECOLOGIA DE *V. vulnificus*

V. vulnificus es una especie natural de estuarios y zonas salobres que se distribuye mundialmente dependiendo de la salinidad y temperatura del agua. Se han descrito factores adicionales que pueden afectar la abundancia de *V. vulnificus* en el agua, como son la limitación de nutrientes, la presencia de bacteriófagos o incluso la de bacterias competidoras.

Estados de inanición y de viabilidad pero no cultivabilidad

En la situación de estrés que supone la limitación de nutrientes, la saturación de oxigeno, la variación en salinidad o en temperatura etc., las bacterias desarrollan estrategias de supervivencia (o estados metabólicos especializados) que les permiten afrontar los "malos momentos". Por lo general, frente a una limitación de nutrientes las bacterias sintetizan o bien más enzimas degradativos o bien sistemas de captura específicos de substrato como pueden ser los sideróforos. Las bacterias en inanición pueden diferenciarse en formas especializadas de resistencia para mantener su viabilidad. Una de estas formas es la conocida como "estado viable pero no cultivable" (VBNC, Viable But NonCulturable). En este estado, las células son metabólicamente activas pero incapaces de dividirse y multiplicarse cuando se siembran en los medios de cultivo usados para su crecimiento rutinario. De este estado las bacterias pueden volver a la normalidad o "resucitar". *V. vulnificus* entra en estado VBNC cuando se expone a 4ºC y "resucita" cuando aumenta la temperatura, lo que podría explicar por que en los meses de

invierno no es posible aislarlo de lugares donde su presencia está más que comprobada en meses más cálidos. La entrada en el estado VBNC provoca profundos cambios morfológicos y bioquímicos de base genética y regulación todavía por dilucidar.

Biopelículas (biofilm)

Las bacterias son capaces de adherirse a superficies inertes o a la superficie de animales acuáticos formando unas estructuras multicelulares denominadas biofilms (biopelículas). En los biofilms las bacterias se asocian formando microcolonias y se mantienen unidas y protegidas dentro de una matriz compuesta mayoritariamente por exopolisacaridos (EPS). Se ha descrito que la formación de biofilm por *V. vulnificus* es inhibida por la glucosa o por el polisacárido capsular (CPS), este último al inhibir la adherencia a la superficie. Recientemente se han encontrado varias proteínas implicadas en la formación de biofilm por *V. vulnificus* que incluyen enzimas de procesos metabólicos, proteínas implicadas en la respuesta al estrés, movilidad y algunas proteínas regulatorias.

Comunicación célula-célula

El termino "*Quorum Sensing*" (QS) describe la capacidad de los microorganismos de percibir y responder a la densidad bacteriana. El QS permite a las bacterias coordinar su comportamiento y está basado normalmente en la producción y la respuesta a moléculas difusibles que actúan como moléculas señal y que se llaman autoinductores (AI). El funcionamiento del QS es sencillo, los AI difunden por la membrana bacteriana y se unen a su receptor, cuando hay alta densidad bacteriana la concentración del AI es elevada y por lo tanto también la del complejo AI+receptor lo que provoca una cascada de procesos que acaba con la expresión de determinados genes. Hasta el momento se han descrito dos sistemas diferentes de QS, el mediado por acetil-homeserina-lactona (AHL), descrito en *V. fishceri,* y el mediado por un furanosil borato diester, descrito en *V. harveyi.* Este segundo sistema es el que se ha encontrado en *V. vulnificus,* aunque también se ha encontrado en

el genoma de las cepas secuenciadas los genes necesarios para una comunicación mediada por AHL, pero, por el momento, no se ha encontrado la molécula difusora.

INFECCIÓN POR *V. vulnificus*

La palabra latina "vulnificus" expresa con propiedad la infección provocada por esta especie en el hombre ya que su traducción es "lo que provoca heridas". La especie se describió en 1979, pero probablemente las infecciones humanas causadas por este microorganismo ya fueron descritas por Hipócrates (460-377 A.C) en su libro Epidemia I. *V. vulnificus* posee una de las tasas más altas de mortalidad entre los patógenos alimentarios conocidos y hoy por hoy es considerado un problema de seguridad alimentaria en varios países, entre ellos miembros de la Unión Europea, Japón, Nueva Zelanda, República de Corea y Estados Unidos de América.

Vibriosis humanas

Se han descrito casos de infecciones humanas debidas a *V. vulnificus* en muchos países del mundo, preferentemente en aquéllos localizados en las zonas templadas y tropicales del planeta. Recientemente, el números de casos en países más fríos, como los países nórdicos, se ha incrementado de forma sustancial por el calentamiento global del planeta. La CDC estima que el número real de infecciones humanas caudas por esta bacteria es muy superior al que queda registrado fundamentalmente por fallos en el diagnóstico al no aislarse el agente causal o, si se aísla, al identificarse de forma incorrecta.

V. vulnificus puede provocar tres tipos de enfermedades:

- Gastroenteritis. Suelen ser infecciones leves que no requieren tratamiento y que se dan tras la ingesta de marisco crudo. Algunos autores ponen en duda la relación entre gastroenteritis y la infección por *V. vulnificus* e indican que en los casos descritos no se

han buscado otras causas y en algunos casos se han aislado otras bacterias además de *V. vulnificus.*

- Septicemia primaria. Se describe como una enfermedad sistémica caracterizada por fiebre o shock sistólico (presión arterial por debajo de 90 mm de Hg) en la cual se aísla *V. vulnificus* de la sangre u órganos internos, estériles en pacientes sanos, sin que exista una herida infectada antes de la enfermedad. Por lo general la infección es precedida por el consumo de marisco (sobre todo ostras) crudo o poco cocinado. En la mayoría de los casos registrados, el paciente padece una enfermedad que le predispone a sufrir la septicemia, como aquéllas que causan una elevación en los niveles de hierro en sangre o aquéllas que alteran el funcionamiento del sistema inmunitario. Uno de los síntomas externos más característico de la septicemia por *V. vulnificus* es la aparición de lesiones secundarias en las extremidades, produciéndose ampollas rellenas de líquido hemorrágico que en ocasiones derivan en ulceras necróticas o incluso gangrenosas obligando a la intervención quirúrgica para limpiar o amputar la zona.
- Infecciones de heridas. Generalmente se producen por la infección de una herida por agua de mar o al herirse manipulando pescado o marisco. La infección, progresa rápido provocando una fascitis necrotizante. En algunos casos la infección puede derivar en septicemia secundaria.

Vibriosis en peces

V. vulnificus biotipo 2 puede causar enfermedad en peces y mariscos (gambas y langostino), sobre todo en cultivo, pero es la vibriosis de la anguila la enfermedad más frecuente y más importante económicamente. La vibriosis de la anguila cursa en forma de brotes o epizootias de alta mortalidad que afectan a

anguilas en cultivo en agua de mar o dulce en todo el mundo. Se caracteriza por ser una septicemia primaria con hemorragias externas e internas afectando los órganos vitales como el hígado, páncreas, riñones o bazo. Los primeros casos se describieron en anguila japonesa entre 1975 y 1977 en Japón. En 1989 se describe por primera vez en Europa, en concreto en España y se extiende en 1990 por todo el continente europeo afectando la producción en Dinamarca, Suecia, Suiza y Reino Unido.

FACTORES DE VIRULENCIA

Se han realizado numerosos estudios sobre los factores de virulencia de *V. vulnificus* relacionados con patogenicidad para el hombre y animales y la principal conclusión que se obtiene es que la virulencia de esta especie no se explica por la producción de una toxina como ocurre con *V. cholerae* o *V. parahaemolyticus*, sino que es un proceso multifactorial en el que juega un papel fundamental la resistencia a las defensas innatas del hospedador, sea éste un hombre o un pez. Con la obtención de mutantes se ha podido demostrar que la cápsula, la presencia de un sistema funcional de génesis flagelar y la síntesis de sideróforos son esenciales para la virulencia. Otra peculiaridad de esta especie es que los factores de virulencia descritos hasta el momento se han encontrado en todos los aislados, incluidos los ambientales, por lo que todos los aislados se consideran que tienen potencial infectivo o infeccioso. Los estudios se han realizado sobre todo en el biotipo 1 y, en el caso del biotipo 2, en la serovariedad E, y han revelado un gran parecido entre ambos biotipos lo que hace pensar que ocurrirá lo mismo con el biotipo 3.

Polisacárido capsular (CPS)

Se ha definido como el factor de virulencia más importante en *V. vulnificus*. Los aislados encapsulados tienen una morfología colonial opaca (Op) mientras que las bacterias que crecen formando colonias traslúcidas (Tr) producen menor cantidad de CPS. La producción de CPS se ha correlacionado con la virulencia para

ratón y anguila, la producción de citoquinas y la resistencia a la fagocitosis y la opsonización. Se ha descrito que la producción de CPS está sometida a regulación aleatoria por variación de fase de forma que con una frecuencia relativamente alta, la bacteria pasa de fenotipo capsulado a no capsulado y con menor frecuencia el cambio inverso. Recientemente se ha descrito una tercera variante colonial, variante rugosa, aislada desde una colonia opaca aunque los autores no indican a que se debe esta rugosidad, si a un cambio en el CPS o en el lipopolisacárido. La variante rugosa es altamente resistente al complemento.

Lipopolisacárido (LPS)

Los síntomas asociados a la septicemia primaria así como la inflamación que se produce en las heridas son, en parte, debidos a la actividad endotóxica del LPS. La cadena lateral del antígeno O de la serovariedad E determina la resistencia al efecto bactericida del suero de anguila no inmunizada.

Adhesinas

Las adhesinas son proteínas de la superficie bacteriana que permiten la unión a receptores localizados en la superficie de las células del hospedador. Existen dos tipos de adhesinas, las fimbriales localizadas en fimbrias o pilis y las no fimbriales localizadas en al superficie celular. Hace tiempo se describió en *V. vulnificus* una estructura tipo pili que se asociaba a la adherencia a celúlas Hep-2. Posteriormente se ha demostrado al presencia de un pili tipo IV que esta relacionado no solo con la adherencia sino con la virulencia para ratón y también con la formación de biofilm.

Flagelo

V. vulnificus como el resto de los componentes del género *Vibrio* posee flagelo polar que le proporciona la movilidad necesaria para penetrar por al mucosa y acceder a las células epiteliales. Mediante la generación de mutantes en genes del flagelo, se ha

conseguido reducir la movilidad de *V. vulnificus* así como también su adhesión, citotoxicidad y grado de virulencia para ratón.

Uso del hierro

Los requerimientos de hierro para diferentes procesos biológicos se extienden a casi todos los seres vivos conocidos. Debido a su baja solubilidad y a su alta toxicidad los organismos superiores han desarrollado sistemas de almacenaje del hierro acomplejados con proteínas como la hemoglobina, transferrina o la ferritina. A su vez, las bacterias han desarrollado sistemas para conseguir hierro, uno de los cuales se basa es la síntesis de sideróforos. *V. vulnificus* puede obtener el hierro necesario para su crecimiento por:

- Síntesis de sideróforos que compiten con las proteínas quelantes del hierro del hospedador (transferrina y lactoferrina).
- Uniendo los grupos hemo de la hemoglobina y otros compuesto hémicos mediante la síntesis de receptores específicos para estas proteínas.

Se han descrito dos tipos de sideróforos, los de tipo fenolato y los de tipo hidroxamato. *V. vulnificus* produce ambos, pero en el biotipo 1 sólo se ha caracterizado el de tipo fenolato (vulnibactina) que es capaz de secuestrar el hierro de la trasferrina humana. En cambio, cuando el biotipo 2 se crece con transferrina férrica como única fuente de hierro produce sideróforos de tipo hidroxamato. Quizá este tipo de sideróforos tiene un papel importante en la virulencia para peces.

Hemolisinas

Las hemolisinas son proteínas que forman poros en los eritrocitos liberando la hemoglobina. En algunos casos su actividad lítica se extiende a otros tipos celulares como sucede con la hemolisina más importante de *V. vulnificus*, la hemolisina/citolisina

VvhA. Esta tóxina es capaz de provocar la apoptosis celular y la producción de oxido nitroso (NO) que contribuye al daño tisular. Se han descrito otras hemolisinas, como la VllY o la HlyIII y en el genoma hay varias posibles pautas abiertas de lectura (ORF) que tienen alta homología con otras citolisinas o hemolisinas bacterianas descritas. Esto puede explicar por que la mutación en el gen *vvhA* no reduce la virulencia, ya que otra hemolisina sustituye a la mutada.

Metaloproteasa

La metaloproteasa Vvp de *V. vulnificus* es una proteasa extracelular que requiere zinc para su actividad catalítica. Posee actividad proteasa frente a un gran número de sustratos como el colágeno, elastina y la caseína. Se ha propuesto que la metaloproteasa es la responsable de las lesiones en la piel debido a que la inyección de la proteína purificada incrementa la permeabilidad vascular que provoca el edema, además de provocar daños hemorrágicos al digerir la membrana vascular. Pese a esto no hay una atenuación de la virulencia para ratón o citotoxicidad cuando esta proteína se muta.

Otros factores de virulencia

Recientemente se ha descrito que *V. vulnificus* posee los genes necesarios para la producción de toxinas tipo RTX, pero no existen muchos datos de su papel real en la virulencia.

Además de los enzimas descritos antes, *V. vulnificus* es capaz de exportar un gran número de enzimas como mucinasas, lipasas, condroitinasas, hialuronidasas, DNasas, esterasas y sulfatasas. Estos enzimas pueden ayudar en la colonización e invasión pero su papel en la virulencia es desconocido.

Regulación de la virulencia

La gran variedad de factores de virulencia que produce *V. vulnificus* se traduce en la existencia de múltiples sistemas para su regulación. Por ejemplo, los sistemas de captación de hierro están regulados por la proteína Fur (Ferric uptake regulator); la respuesta de la bacteria frente a la disponibilidad de fuentes de carbono está regulada por represión por catabolito mediada por cAMP que, además, regula una gran cantidad de genes, entre ellos de virulencia; la respuesta al estrés también tienen un papel regulador de la virulencia; y finalmente, el QS regula la expresión de varios genes entre ellos la virulencia ya que se ha visto que afecta la síntesis de hemolisinas y proteasas.

Susceptibilidad del hospedador

V. vulnificus es una especie particularmente virulenta que, una vez infecta, causa una alta tasa de mortalidad. Sin embargo, la infección en humanos suele estar asociada a la existencia de condiciones que favorecen la multiplicación de la bacteria. Estos factores de riesgo suelen ser enfermedades crónicas que afectan o al hígado a al sistema inmunitario. Una característica en común de las enfermedades hepáticas es un aumento considerable de los niveles de hierro en sangre, que favorece el crecimiento bacteriano y además impide la fagocitosis mediada por anticuerpos.

Con respecto a los peces, no parecen existir ningún factor que predisponga a la infección, de hecho la vibriosis es fulminante cuando se dan episodios epizoóticos en instalaciones de acuicultura por primera vez.

MODELOS PARA EL ESTUDIO DE LA INFECCIÓN POR *V. vulnificus*

Cultivos celulares

Los cultivos celulares se han usado en el estudio de la patogénesis de *V. vulnificus* pero centrados sobre todo en la evaluación de los efectos citopáticos de enzimas y toxinas sobre determinadas líneas celulares.

Roedores

Ratones y ratas son los modelos animales más usados para el estudio de la infección de *V. vulnificus* en humanos. A menudo es necesario un tratamiento previo con alguna forma férrica que mimetice las condiciones que predisponen a la infección. Los daños histológicos observados con la inoculación de la bacteria son similares a los observados en los casos clínicos exceptuando las lesiones en la piel tan características de *V. vulnificus.*

Anguila

Como hospedador natural es el mejor ejemplo para estudiar la infección por *V. vulnificus.* El patógeno entra en la anguila usando las agallas como punto de entrada a la sangre, extendiéndose así por todos los órganos internos donde la bacteria persiste. La vacunación ha permitido el estudio de la respuesta inmunitaria frente a la infección por *V. vulnificus* demostrando que hay dos compartimentos inmunitarios separados, uno mucosal y otro sistémico, con diferentes cinéticas en la producción de anticuerpos.

Otros modelos

El nematodo *Caenorhabditis elegans* esta siendo usado como modelo de uso cómodo y barato para el estudio de la patogénesis de varios patógenos incluidos *V. vulnificus.* Y aunque no se ha usado todavía, el pez cebra (*Danio rerio*) ofrece muchas posibilidades para

el estudio de los factores de virulencia ya que se conoce su genoma y se pueden obtener mutantes que desarrollen las distintas condiciones o enfermedades que predisponen para la enfermedad.

OBJETIVOS

Este trabajo se incluye dentro de la investigación realizada por el grupo "Patógenos de animales acuáticos con interés en Salud Publica" del Departamento de Microbiología y Ecología de la Facultad de Biología de la Universidad de Valencia. Este grupo ha trabajado en el estudio de la biología de *V. vulnificus*, centrándose en las cepas patógenas de peces (biotipo 2). Dentro de esta línea de investigación se propuso el presente proyecto de Tesis Doctoral con el nombre "Epidemiología y filogenia del patógeno *V. vulnificus* biotipo 2".

El objetivo principal de la presente Tesis Doctoral fue averiguar cómo habían aparecido los clones virulentos para peces a lo largo de la historia evolutiva de *V. vulnificus*. Con este propósito, primero ampliamos la diversidad genética de nuestra colección de cepas, para lo que recogimos cepas de los tres biotipos de todo el mundo y desarrollamos un protocolo de aislamiento específico que nos permitiera obtener aislados ambientales del biotipo 2 hasta el momento inexistenes. Una vez consolidada la colección, analizamos la variabilidad intraespecífica de *V. vulnificus* aplicando metodologías genotípicas y fenotípicas y construimos un árbol filogenético basándonos en la secuenciación parcial de genes de mantenimiento (*housekeeping*) y de virulencia. En paralelo, aplicamos el conocimiento obtenido en estos estudios al desarrollo de nuevas metodologías para el diagnóstico de la vibriosis así como la identificación rápida del patógeno y de portadores sanos.

RESULTADOS Y CONCLUSIONES

DESARROLLO DE UN PROTOCOLO ESPECIFICO DE AISLAMIENTO DE *V. vulnificus* SEROVAR E A PARTIR DE MUESTRAS AMBIENTALES.

El objetivo principal de este apartado fue el desarrollo de un protocolo en dos pasos que permitiera aislar cepas de la serovariedad E a partir de muestras de agua y animales portadores. Este nuevo protocolo debería favorecer el crecimiento de la serovariedad E, e inhibir el de sus competidores, entre ellos el biotipo 1, que puede ser co-aislado junto con el biotipo 2 de muestras ambientales.

Resultados

Eficiencia del caldo de enriquecimiento. Se diseñó un caldo de enriquecimiento selectivo, el caldo SEB, que contiene suero fresco de anguila como única fuente de nutrientes para crecer. En este caldo el crecimiento de las cepas de serovariedad E fue significativamente superior al de sus competidores, incluyendo el biotipo 1, especialmente cuando la salinidad se ajustaba al 1%. Se propuso como protocolo de pre-enriquecimiento el uso del caldo SEB-1 y una incubación de 8 horas. La eficacia selectiva de este caldo se valoró con una colección de cepas de *V. vulnificus* y otras especies, encontrándose crecimiento positivo sólo cuando se inoculaban las cepas de *V. vulnificus* biotipo 2.

Eficiencia del medio selectivo. De los distintos medios selectivos propuestos para el aislamiento de *V. vulnificus*, sólo el medio VVM rindió resultados aceptables para la recuperación no sólo de la serovariedad E, sino también del resto de grupos de *V. vulnificus*, especialmente el biotipo 3. Entre las cepas de otras especies, sólo *V. mediterraneii* creció en este medio, aunque dio colonias distintas a las de *V. vulnificus*.

Aplicación del protocolo en muestras ambientales. El protocolo final de enriquecimiento en SEB-1 durante 8 horas y siembra posterior en el agar selectivo VVM se realizó con varias muestras de agua de mar y de tanques de anguila, así como de anguilas sanas. Como protocolo control se usó la siembra en agua alcalina de peptona (APW) seguida de siembra en VVM. Con ambos protocolos se pudo aislar *V. vulnificus* de las muestras de agua y tejido pero sólo cuando se usó SEB-1 se encontraron aislados de biotipo 2 y serovariedad E. Algunos de estos aislados fueron estudiados en profundidad y comparados con la cepa control CECT 4604 (serovariedad E) obteniéndose resultados similares tanto en el estudio bioquímico como en la valoración de la supervivencia en suero de anguila y humano y el grado de virulencia para ratón y anguila.

Conclusión

Se ha desarrollado un nuevo protocolo para el aislamiento específico de la serovariedad E de muestras ambientales y, posteriormente, se ha validado su uso en muestras naturales de agua y portadores sanos. El elemento clave del protocolo es el paso del enriquecimiento selectivo, en el que se hace uso de la resistencia específica de las cepas de serovar E al suero de la anguila para conseguir un mayor crecimiento de éstas en detrimento de sus competidoras, que se ven inhibidas. En el segundo paso, se seleccionó un medio selectivo y diferencial, de los múltiples propuestos para *V. vulnificus*. En este medio la eficiencia de recuperación es mucho mayor para todas las cepas y grupos de *V. vulnificus* estudiados. El uso de este protocolo con muestras naturales permitió por primera vez aislar la serovariedad E de muestras ambientales, en ausencia de anguilas y de epizootias, resultado que confirma que estas cepas tienen su reservorio en el ambiente acuático. Además, estos nuevos aislados ambientales son potencialmente virulentos para el hombre y para peces por lo que deben tenerse en cuanta en los estudios epidemiológicos.

IDENTIFICACION DE SECUENCIAS DE ADN ESPECÍFICAS DE *V. vulnificus* BIOTIPO 2 MEDIANTE HIBRIDACIÓN SUBSTRACTIVA SUPRESORA

La hibridación substractiva supresora (SSH) es una metodología que permite comparar los genomas de dos muestras diferentes y revelar las diferencias entre ellos. Nosotros aplicamos esta técnica con la intención de encontrar secuencias de ADN que fueran específicas de la serovariedad E (*tester*) y, por tanto, ausentes la mezcla de ADN correspondiente a varios aislados de biotipo 1 (*driver*). Es de esperar que en estas secuencias se encuentre algún factor de virulencia que explique el carácter virulento para peces exclusivo del biotipo 2.

Resultados

Substracción genómica entre el biotipo 1 y la serovariedad E de *V. vulnificus.* La técnica SSH se empleó para comparar la cepa CECT 4601 de biotipo 2 y serovariedad E, aislada de anguila enferma, con tres cepas de biotipo 1 todas ellas de sangre humana; CS9133, YJ016 y CECT 529^T (la cepa tipo de la especie). De los 85 clones obtenidos al principio, sólo 8 de ellos no dieron reacción cruzada y fueron específicos para la serovariedad E. Estos fragmentos se secuenciaron y compararon con la base de datos encontrándose homología en 4 de ellos; 2 poseían una alta homología con posibles transposasas de *Listonella anguillarum* (CT005 y CT010), y los otras dos eran similares a supuestas proteínas de *Aeromonas salmonicida* (CT012) o *V. parahaemolyticus* (CT067). Cinco de estas secuencias se comprobó que estaban localizadas en un plásmido de alto peso molecular que comparten todas las cepas de biotipo 2, mientras que el resto estaban localizadas en el cromosoma.

Distribución de las secuencias específicas. Para poder averiguar si las secuencias encontradas por SSH eran realmente específicas del biotipo 2 se diseñaron cebadores y se realizó la reacción en cadena de la polimerasa (PCR) con una colección de

cepas que incluía aislados de los distintos biotipos junto con cepas de otras especies. Dos de estas secuencias (CT005 y CT012) fueron descartadas al dar resultados negativos en algunos aislados de biotipo 2 y positivos en otros grupos de *V. vulnificus*. Del resto se encontró que las 3 plasmídicas (las otras dos fueron las descartadas) eran compartidas por todas las cepas de biotipo 2, independientemente de la serovariedad, mientras que las cromosómicas estaban sólo en las cepas que pertenecían a la serovariedad E.

Conclusión

Usando la técnica del SSH hemos podido obtener secuencias de ADN que nos permiten diferenciar las cepas virulentas para la anguila de las que no lo son y dentro de las virulentas aquéllas que pertenecen a la serovariedad con potencial zoonótico, que es la serovariedad E. La presencia de las secuencias específicas del biotipo 2 en plásmidos sugiere que la virulencia para anguilas puede haber sido adquirida por estos organismos mediante transferencia genética horizontal desde un donador desconocido. Por ultimo, las secuencias obtenidas en este estudio pueden ser utilizadas para el diseño de un método molecular de identificación y diagnostico.

PCR MULTIPLE PARA LA DETECCION DE *V. vulnificus*. BIOTIPO 2

Siguiendo con el trabajo anterior se planteó el objetivo de desarrollar una metodología molecular de biotipado que permitiera la identificación de los aislados patógenos para los peces (biotipo 2) y al mismo tiempo discriminar aquéllos con potencial virulento para el hombre (serovariedad E). Como objetivo secundario se planteó la adaptación del protocolo para el diagnostico de la vibriosis y la detección del patógeno en posibles portadores.

Resultados

Desarrollo de una PCR múltiple. La obtención de secuencias especificas para el biotipo 2 y la serovariedad E en el apartado anterior permitió el diseño de una PCR múltiple que combina tres 3 pares de cebadores; uno específico de especie, que está basado en la secuencia del gen de la hemolisina/citolisina (VvhA) presente en todos las cepas de *V. vulnificus,* y los otros dos específicos de biotipo 2 y serovariedad E.

Uso de la PCR como método de biotipado. Se realizó el biotipado por PCR y por métodos clásicos de la colección de cepas de *V. vulnificus* y se encontró que la correspondencia de ambos procedimientos era del 100%. Se identificaron dos nuevas cepas de serovar E de origen humano, previamente no biotipadas. Ambas fueron fenotípicamente similares a las cepas de anguila y ambientales de la misma serovariedad y también fueron virulentas para peces confirmando la homogeneidad de esta serovariedad.

Uso de la PCR múltiple como método diagnostico. Se contaminó tejido de anguila para determinar la cantidad mínima de bacterias que el método era capaz de detectar obteniéndose niveles (alrededor de 15 UFC/mg de tejido) que permitían el uso de la PCR para el diagnostico. Se utilizó en paralelo la PCR y la detección clásica a partir de anguilas infectadas de forma natural y artificial. En todos los casos el resultado de la PCR fue concordante con el obtenido por metodologías clásicas de diagnóstico.

Uso de la PCR múltiple para la detección de portadores sanos. Muestras de varias anguilas sanas procedentes de piscifactorías se procesaron tanto para el asilamiento de *V. vulnificus* como para su detección por PCR múltiple. Para ello, se realizó primero un enriquecimiento en APW o SEB-1 y luego se sembró en VVM o se procedió a la extracción del ADN y posterior PCR. Dos de las diez muestras aisladas fueron positivas tanto por PCR como por métodos cultivables confirmando que la PCR podía usarse para la detección de portadores sanos.

Conclusión

Se ha desarrollado una valiosa herramienta para la identificación rápida del patógeno de peces *V. vulnificus* biotipo 2 que consiste en un protocolo de PCR múltiple que puede aplicarse a muestras tanto de cultivos como de tejidos. En el caso de muestras donde el patógeno se encuentre en minoría (portadores sanos), la PCR se debe utilizar tras un paso previo de enriquecimiento selectivo. El ensayo permite la discriminación de las muestras que pueden suponer un riesgo para la Salud Pública por contener la serovariedad zoonótica. Así mismo, este protocolo puede ser usado en estudios epidemiológicos para biotipar correctamente los aislados clínicos humanos y averiguar la verdadera relevancia de la serovariedad E como patógeno humano. Con esta metodología ha sido posible la identificación de nuevos asilados humanos de serovariedad E procedentes de Francia y Suecia, lo que supone que esta serovariedad constituye un serio riesgo para la Salud Pública.

DIVERSIDAD GENOTIPICA Y FENOTIPICA DE *V. vulnificus.*

En el presente capítulo estudiamos la variabilidad fenotípica y genotípica de la colección de cepas de *V. vulnificus* centrándonos sobre todo en el biotipo 2 de la especie. Los objetivos parciales del presente capítulo fueron: i) evaluar tres sistema rápidos de identificación bioquímica para *V. vulnificus* y sus biotipos y, al mismo tiempo, estudiar la diversidad fenotípica de la especie; ii) encontrar, si fuera posible, algún marcador fenotípico que permitiera diferenciar las cepas según el biotipo al que pertenecen y, iii) validar tres marcadores genéticos de virulencia para humanos así como el ribotipado como técnica de biotipado.

Resultados

Análisis fenotípico. Se usaron los sistemas API 20E, API 20NE y BIOLOG GN2 para la identificación fenotípica rápida de la especie con todos los aislados de la colección. El sistema BIOLOG

fue el que identificó los aislados con mayor precisión (80% de aislados identificados de forma correcta), seguido del API20E (60%) y del API20NE (0%). Los perfiles bioquímicos obtenidos con estos sistemas no se pudieron asociar ni con biotipo, ni con origen del aislado. No obstante, se confirmó que un resultado negativo en la prueba del indol, puede utilizarse para diferenciar los aislados de la serovariedad E con el sistema API20E, y en la utilización de celobiosa, para diferenciar los aislados de biotipo 3 con el sistema BIOLOG.

Estudio de la diversidad genética: polimorfismo en determinadas localizaciones genéticas. Se han descrito varios protocolos de PCR para detectar polimorfismos en diferentes loci génicos que se asocian con la virulencia para humanos. Entre ellos destacan los polimorfismos en el gen de la hemolisina VvhA, en el gen del ARNr 16S y un genotipado (genotipos E y C) basado en resultados obtenidos tras un análisis de RAPD. Aplicando los tres análisis a nuestra colección de cepas hemos encontrado tres perfiles mayoritarios:

- Perfil 1: hemolisina tipo 1, ARNr 16S tipo B y genotipo C. Presentado por aislados de biotipo 1, en su mayoría de sangre humana y de ostra.
- Perfil 2: hemolisina tipo 2, ARNr 16S tipo A y genotipo E. Presentado por aislados con de biotipo 1 del ambiente o de herida humana y aislados del biotipo 2 de cualquier origen y serovariedad.
- Perfil 3: Es una mezcla de los anteriores perfiles; hemolisina tipo 1, ARNr 16S tipo AB y genotipo E. Este perfil es exclusivo de las cepas del biotipo 3.
- Perfiles atípicos: Incluye a todas las cepas que no se ajustan a los anteriores perfiles. En todos los casos son cepas de biotipo 1, en su mayoría ambientales.

Estudio de la diversidad genética: Ribotipado. Usamos el RiboPrinter para realizar el ribotipado con *Hind*III de la colección de cepas de *V. vulnificus*. El árbol UPGMA obtenido muestra la

población de cepas agrupada en dos subdivisiones; la A, que agrupa a cepas de biotipo 1, la mayoría de sangre humana o de ostra y con perfil 1, y la B, más grande, donde están al completo el biotipo 2 y 3 junto con muchas cepas de biotipo 1 ambientales o aisladas de heridas infectadas. Algunos taxones dentro del árbol tienen importancia epidemiológica; por ejemplo todas las cepas del biotipo 3 forman un grupo homogéneo que presenta el mismo ribotipado, al igual que sucede con la mayoría de las cepas del biotipo 2, que aunque presenta más de un ribotipo, la mayoría de las cepas presentaron el mismo.

Conclusiones

Nuestros resultados demuestran que la especie y los tres biotipos, en especial el biotipo 1, son heterogéneos a nivel bioquímico, no correspondiéndose ningún perfil con biotipo, serovariedad u origen del aislado. La división de la especie en biotipos se propuso por diferencias en pruebas bioquímicas entre unos pocos aislados. Sin embargo, la variabilidad bioquímica se pone de manifiesto cuando estas pruebas se realizan en una colección que contiene cepas tanto ambientales como clínicas, y entre éstas, tanto de animales como humanos, de diferentes orígenes geográficos y aisladas durante un largo periodo de tiempo. Los marcadores de virulencia para humanos, basados en polimorfismos de ADN, si bien dividen a la especie en tres grandes grupos que se correlacionan, en parte, con las dos subdivisiones encontradas por ribotipado, no pueden considerarse como predictores de virulencia. Finalmente, la actual división en biotipos no es corroborada por nuestros resultados que, además, demuestran que estas claves bioquímicas no reflejan la verdadera estructura de la especie. La invalidez de los biotipos se ve reforzada con las pruebas genéticas, puesto que en todos los casos la colección se ha dividido en grupos que no tienen correlación con biotipo.

EPIDEMIOLOGIA MOLECLAR Y COMPOSICION CLONAL DE LA ESPECIE *V. vulnificus*

Hace cerca de 10 años se propuso el uso de la metodología denominada "Multilocus sequence typing" o MLST para la clarificación de las relaciones epidemiológicas de patógenos humanos. Este sistema de tipado esta basado en la secuenciación de zonas internas de genes de mantenimiento celular y en el agrupamiento de las cepas por secuencias tipo. Desde entonces la metodología se ha aplicado a diferentes especies, no sólo de bacterias, y ha sufrido algunas modificaciones como la inclusión de genes de virulencia entre los genes a secuenciar. Entre los esquemas propuestos existe uno para el estudio del último biotipo propuesto de *V. vulnificus*, el biotipo 3. En esta última parte del proyecto de tesis hemos seleccionado 4 de los 10 genes de mantenimiento celular propuestos en el esquema de MLST para *V. vulnificus* y hemos incluido otros 3 asociados a virulencia para realizar un estudio epidemiológico de la especie *V. vulnificus*, y, en especial del biotipo 2, con nuestra colección de aislados.

Resultados

Diversidad genética observada por MLSA (Multilocus Sequencie Analysis). Se identificaron 73 "sequence types (ST)" diferentes en los 115 *V. vulnificus* analizados, 32 de las cuales pueden organizarse formando clones o complejos clonales. Estos complejos tienes su correspondencia con taxones en las diferentes filogenias que pueden obtenerse de este estudio: los arboles de los 7 genes estudiados solos, o concatenados (todos, solo virulencia o solo mantenimiento). En todos estos árboles las cepas se dividen en dos grandes divisiones como la observada en el ribotipado (y también por otros autores).

Conclusiones

Nuestros resultados han puesto de manifiesto que, pese a la existencia de algunos clones, la estructura de la población no es

clonal ya que la mayoría de los ST están compuestos por una única cepa. Lo que confirma, además, la gran diversidad encontrada dentro de la especie, que ya se observo en el estudio fenotípico. Los complejos clonales están formados o por aislados del biotipo 2 o por aislados del biotipo 3, que son los únicos aislados asociados a procesos de amplificación del clon por brote o epidemia/epizootia registrados entre seres humanos (biotipo 3) o animales (biotipo 2).

Las filogenias obtenidas demuestran claramente que el biotipo 1, ya considerado heterogéneo, esta repartido por todo el árbol y que el biotipo 2, hasta ahora considerado más homogéneo, tiene, en realidad, diferente orígenes filogenéticos dando distintos grupos o clones, con diferencias entre ellos. De hecho, solo la virulencia para peces parece ser el rasgo común a todas estas cepas, propiedad que podrían haber adquirido a la vez que el plásmido de virulencia por lo que sería más propio hablar de patovar que de biotipo.

En general, los datos obtenidos en este trabajo no apoyan la actual división en biotipos de la especie y, consideramos que es necesaria una reclasificación basada en otros criterios. La división en dos grupos observada a lo largo del trabajo, ya sea con los perfiles mayoritarios en el estudio de los polimorfismos, como en las filogenias obtenidas con el ribotipado y con los genes secuenciados puede ser un punto de partida para la propuesta de una nueva división.

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Reunido el Tribunal que suscribe, en el día de la fecha acordó otorgar a esta Tesis Doctoral de

D./Dª. **EVA SANJUÁN CARO**

la calificación de ..Excelente Cum laude.....

Valencia, a ...13. de .....marzo..... de ...2008.....

EL/LA PRESIDENTE/A

EL/LA SECRETARIO/A,

Dra. Dª. Belén Fouz Rodríguez

Dra. Dª. Esperanza Garay Aubán