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Facultat de Ciències Biològiques
Departamento de Microbiología y Ecología

PROGRAMA DE POSTGRADO EN BIOTECNOLOGÍA

IRON AND VIRULENCE IN THE ZOONOTIC PATHOGEN *Vibrio vulnificus*

Memoria presentada por
David Pajuelo Gámez
para optar al grado de
Doctor en Ciencias Biológicas
por la Universidad de Valencia

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Dirigida por la **Dra. Carmen Amaro González**

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Y para que así conste a los efectos oportunos, firma la presente en Valencia en Octubre de 2013.

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Albert Einstein

La verdad está ahí fuera.

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ABBREVIATIONS

ABBREVIATIONS

ABCt: ATP binding cassette transporter

Abs$_x$: Absorbance at x nm

ASW: Artificial sea water

ATCC: American Type Culture Collection

BLAST: Basic local alignment search tool

BSA: Bovine serum albumin

Bt: Biotype

CAS: Chrome azurol S

cDNA: Complementary DNA

CECT: Colección Española de Cultivos Tipo

CFU: Colony-forming units

cRNA: Complementary RNA

CRP: cAMP receptor protein

CTAB: Cetyl trimethylammonium bromide

Cy3: Cyanine 3 dye

DEPC: Diethyl pyrocarbonate

dNTP: Deoxynucleotide triphosphate

DTT: Dithiothreitol

ECP: Extracellular products

EDDHA: Ethylenediamine-di-[o-hydroxyphenylacetic] acid

ELISA: Enzyme-linked immunosorbent assay

EP: Eel plasma

Fur: Ferric uptake regulator

FURTA: Fur titration assay

h: Hour

Hb: Hemoglobin

HBSS: Hank's balanced salt solution

Hm: Hemin

HP: Human plasma

IROMP: Iron regulated outer membrane proteins

kb: Kilobase

ABBREVIATIONS

KDa: Kilodalton

LD$_{50}$: Lethal dose 50

LDH: Lactate dehydrogenase

LPS: Lipopolysaccharide

MARTX: Multifunctional autoprocessative repeat in toxin

MIC: Minimum inhibitory concentration

Min: Minutes

MLSA: Multilocus sequence analysis

moi: Multiplicity of infection

OD$_{600}$: Optical density at 600 nm

OMP: Outer membrane protein

ORF: Open reading frame

bp: Base pair

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PEC: Peritoneal exudate cells

pR99: Virulence plasmid of R99 (CECT4999) strain

PVDF: Polyvinylidene fluoride

pVvBt2: Generic virulence plasmid of biotype 2 strains

qPCR: Quantitative Polymerase chain reaction

qRT-PCR: Quantitative reverse-transcription Polymerase chain reaction

QS: Quorum sensing

RT: Room temperature

RTX: Repeat in toxin

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis

sSNP: Synonymous single nucleotide polymorphism

TD$_{50}$: Toxic dose 50

Tf: Transferrin

VvBtSerE: *Vibrio vulnificus* biotype 2 serovar E

SUMMARY

SUMMARY

Vibrio vulnificus es un patógeno humano emergente que es autóctono de ecosistemas acuáticos salobres de climas templados, tropicales y subtropicales. La especie se subdivide en 3 biotipos (Bt), de los cuales el Bt2 contiene un grupo de cepas que, además de poder infectar al hombre, pueden causar infecciones en peces. Este grupo es un complejo clonal serológicamente homogéneo que denominamos serovar E o serovar zoonótica (VvBt2SerE). Como patógeno humano, VvBt2SerE causa casos esporádicos de infecciones graves en heridas que pueden derivar en septicemia secundaria en pacientes inmunocomprometidos y como patógeno de peces, brotes o epizootias de una septicemia hemorrágica conocida como vibriosis de aguas cálidas. La presente Tesis se centra en averiguar el papel en la virulencia de VvB2SerE de genes seleccionados en base al conocimiento que existe sobre los otros biotipos de la especie, en especial el Bt1, y su patogenicidad para humanos. En concreto, se han seleccionado los genes *rtxA1₃*, *hupA*, *hutR, vuuA*, *vep20* y *fur*. Los resultados más relevantes que se han encontrado, se discuten a continuación.

rtxA1₃ codifica una toxina de la familia MARTX (Multifunctional Autoprocessatve Repeat in Toxin) única en la especie (tipo III) que en el Bt1 (tipo I) está relacionada con invasión y resistencia a la fagocitosis. Nuestro trabajo demuestra que la toxina tipo III ejerce la misma función que la tipo I en mamíferos y una función diferente en peces. Encontramos que la mutación del gen no produce efectos en la capacidad de colonización e invasión de VvBt2SerE pero anula su virulencia para anguila, lo que unido a que el patógeno causa la muerte sin alcanzar los tamaños poblacionales en órgano interno propios de otros vibrios, sugiere que la toxina produce la muerte de los animales por choque tóxico. Demostramos que el gen *rtxA1₃* se expresa *in vivo* y que se activa sólo tras el contacto directo de la bacteria con células eucarióticas y lo relacionamos con citotoxicidad para distintos tipos celulares, incluyendo células de defensa (eritrocitos y neutrófilos) por lo que hipotetizamos que el choque tóxico se produce porque la toxina desencadena una tormenta de citoquinas como consecuencia de la interacción de la bacteria con las células de defensa. Asimismo, pudimos relacionar la toxina con resistencia a predación por amebas y ampliar su papel de factor de virulencia a factor de supervivencia fuera del hospedador, lo que explicaría por qué el gen *rtxA1₃* está presente en todos los clones y complejos clonales del Bt2 y por duplicado, en plásmido y cromosoma.

SUMMARY

hupA, *hutR* y *vuuA* son tres genes cromosómicos que en el Bt1 codifican para tres receptores relacionados con crecimiento en condiciones restrictivas en hierro. *vep20* es un gen plasmídico, no estudiado, que presenta homología con receptores para hemina/hemoglobina y transferrina. Este trabajo relaciona *hupA* con captación de hemina (*hutR* es un gen secundario) y *vuuA* con captación de vulnibactina por VvBt2SerE y demuestra que la anulación del sistema de captación de hemina dependiente de HupA o del sistema de captación de vulnibactina por mutación de los receptores reduce la virulencia para peces y mamíferos mientras que la anulación de ambos sistemas atenúa aún más la virulencia para peces y elimina completamente la virulencia de la bacteria para mamíferos. Además, los resultados obtenidos con el mutante en *vep20* sugieren que hay un tercer sistema de captación de hierro en la serovariedad zoonótica, esta vez plasmídico, que probablemente depende del reconocimiento de una proteína almacenadora de hierro o, lo más probable, del quelante transferrina, específicamente para peces. La secuenciación de los tres genes en una amplia colección de cepas de la especie y su posterior análisis filogenético demuestra que *hupA* y *vuuA* son genes antiguos que pertenecen al *core* de la especie y que presentan un grado de variación indicativo de presión de selección relacionada con procedencia del aislado (competencia por sideróforos, adaptación a hemoglobina de los peces...) mientras que *vep20* es un gen de adquisición reciente y no presenta variación.

Finalmente, dada la importancia que el hierro tiene en la virulencia de esta especie para peces y mamíferos, hemos obtenido un mutante en el gen regulador *fur*, que hemos caracterizado fenotípicamente y valorado usando un microarray diseñado específicamente para VvBt2SerE. Los resultados preliminares confirman que hay cientos de genes regulados por Fur, de forma dependiente o no de hierro, y regulados por hierro, y que Fur, además de un represor, puede actuar como activador y que, en conjunto, controlan funciones tan dispares como: movilidad, quimiotaxis, producción de cápsula y lípido A, resistencia a péptidos microcidas y a formas reactivas del oxígeno, resistencia al suero, al choque térmico, etc. Parte de estas funciones han sido confirmadas diseñando experimentos y comparando las diferencias entre cepa parental y mutante o entre condiciones de crecimiento, con y sin hierro.

SUMMARY

En conclusión, VvBt2SerE posee un set de genes que le capacita tanto para sobrevivir en el medio ambiente como para infectar hospedadores tan distintos como peces y mamíferos, que contribuyen directamente a la colonización, invasión y destrucción de los tejidos/órganos del hospedador, siendo este proceso y otros tantos regulados por la concentración de hierro y el regulador global Fur.

SUMMARY

Vibrio vulnificus is an emerging human pathogen that inhabits aquatic ecosystems in temperate, tropical and subtropical climates. The species is subdivided in three biotypes (Bt), of which the Bt2 comprises a group of strains that can infect both human and fish. This group is a clonal complex, serologically homogeneous, denominated serovar E or zoonotic serovar (VvBt2SerE). As human pathogen, VvBt2SerE causes sporadic cases of wound infections that can derive to secondary septicemia in immunocompromised patients, and as fish pathogen causes outbreaks of a primary septicemia known as warm-water vibriosis. The present Thesis is focused on find out the role in the VvBt2SerE virulence of selected genes, chosen in basis of the other biotypes of the species, specially the Bt1, and its pathogenicity for humans. Specifically, the genes *rtxA1₃*, *hupA*, *hutR, vuuA*, *vep20* and *fur* were selected. The results are discussed below.

rtxA1₃ codifies for a toxin of the MARTX family (Multifunctional Autoprocessatve Repeat in Toxin) exclusive in the species (type III) that in the Bt1 (type I) is involved in invasion and resistance to phagocytosis. Our work demonstrates that type III toxin exerts the same function that type I in mammals but a different one in fish. We found that gene mutation did not produce effects in colonization and invasion of the VvBt2SerE but abolished the virulence for eels, and considering that the pathogen causes the animal death without rising a high number population common in other *Vibrio* species, the results suggest that the toxin produce the animal death by toxic shock. We demonstrated that the gene *rtxA1₃* is expressed *in vivo* and only when the bacteria is in direct contact with eukaryotic cells, and that present a high cytotoxic activity towards different cellular types, including cells of the immune system (erythrocytes and neutrophils), so we hypothesized that the toxic shock is produced because the toxin triggers a cytokine storm as consequence of the interaction with immune cells. Moreover, we could relate the toxin with the resistance to amoeba predation and extend its role of virulence factor as a survival factor outside the host, what is according with the fact that the gene *rtxA1₃* is present in all clones and clonal complexes of the Bt2 and by duplicated, in the plasmid and the chromosome.

hupA, *hutR* and *vuuA* are chromosomic genes that in the Bt1 codify for three receptors related with growth in iron restricted conditions. *Vep20* is an

uncharacterized plasmidic gene that present homology with hemin/hemoglobin and transferrin receptors. This work relates *hupA* with the use of hemin (*hutR* is a secondary heme-receptor) and *vuuA* with the use of ferric vulnibactin by VvBt2SerE and demonstrate that the inactivation of the HupA- or VuuA-dependent systems reduce the virulence degree for fish and mammals while the inactivation of both decreases even more the virulence degree for fish and abolishes completely the virulence for mammals. Moreover, the results obtained with the *vep20* mutant suggest that there is a third iron acquisition system in the zoonotic serovar, in this case plasmidic, that is probably involved in the scavenge of iron from transferrin, specifically for fish. The sequencing of the three genes in a wide group of strains and the phylogenetic analysis demonstrate that *hupA* and *vuuA* belong to the *core* genes of the species and present sequence variability, while *vep20* is a recently acquired gene without variation.

Finally, given the importance of iron in the virulence of this species for fish and mammals, we obtained a mutant in the *fur* gene to characterize it phenotypically and use it in a microarray designed specifically for VvBt2SerE. The preliminary results confirm that there are hundreds of genes under control of Fur and iron, and that Fur can also work as an activator in addition to as a repressor, controlling phenotypes such as: motility, chemotaxis, capsule and lipid A synthesis, resistance to microcide peptides, plasma, heat shock, etc. Part of these functions have been confirmed with phenotypic assays by comparing the effects of *fur* mutation and the presence/absence of iron.

In conclusion, VvBt2SerE possesses a set of genes that enables both survive in the environment and infect different hosts such as fish and mammals, contributing directly to the colonization, invasion and destruction of hosts tissues/organs, being this process and many others regulated by the iron concentration and the global regulator Fur.

HYPOTHESIS

AND

OBJECTIVES

HYPOTHESIS

1. *rtxA1₃*, *hupA*, *hutR*, *vuuA* **and** *vep20* **are virulence factors for** *V. vulnificus* **biotype 2 serovar E.**

2. Iron, throughout Fur and/or other regulators controls virulence in *V. vulnificus* **biotype 2 serovar E.**

OBJECTIVES

1. To find out the role of **MARTX type III** (encoded by *rtxA1₃*) in the virulence and survival of *V. vulnificus* biotype 2 serovar E.

    Milestone 1. To study the expression of *rtxA1₃* and get single and double mutants (the gene is duplicated in chromosome II and plasmid) and the corresponding complemented strains.

    Milestone 2. To determine the virulence degree as well as the colonization and invasion degree of the mutant and complemented strains and compare them with that of the wild-type strain.

    Milestone 3. To determine the role of *rtxA1₃* in the interaction bacteria/eukaryotic cells by using primary cultures and cell lines of epithelial and defensive cells from fish and mammals, and including amoeba isolated from fish at cellular level.

2. To investigate the role of ***hupA, hutR, vuuA*** and ***vep20*** in the virulence of *V. vulnificus* biotype 2 serovar E as well as to determine the phylogeny of each gene.

> ***Milestone 1***. To study the expression ***hupA, hutR, vuuA*** and ***vep20*** and get single and multiple mutants, and the corresponding complemented strains.

> ***Milestone 2.*** To determine the virulence degree as well as the colonization and invasion degree of the mutant and complemented strains and compare them with that of the wild-type strain.

> ***Milestone 3***. To determine the role of ***hupA, hutR, vuuA*** and ***vep20*** in the growth of the bacterium in plasma and different iron-deficient media.

> ***Milestone 4.*** To sequence the genes in a wide collection of strains of the species and analyze phylogenetically the sequences.

3. To determine the regulon **Fur** and **iron** in *V. vulnificus* biotype 2 serovar E**.**

> ***Milestone 1.*** To design and validate a **microarray platform** containing oligoprobes for all the ORF identified in the genome of the strain of VvBt2SerE CECT4999.

> ***Milestone 2***. To get a *fur* defective mutant and its complemented strain and test their phenotype with respect to the wild-type strain

> ***Milestone 3***. To identify the whole **Fur** regulon and **iron**-regulon by using the microarray platform.

> ***Milestone 4.*** To test the veracity of the microarray results by performing a selection of specifically-designed experiments.

INDEX

INTRODUCTION

CHAPTER 1: MARTX OF THE ZOONOTIC SEROVAR OF *Vibrio vulnificus*

CHAPTER 2: IRON ACQUISITION SYSTEMS OF THE ZOONOTIC SEROVAR OF *Vibrio vulnificus*

INTRODUCTION

I.   *Vibrio vulnificus* and its zoonotic variant

A.   Taxonomic context: biotypes and serotypes

Vibrio vulnificus is a gram-negative, oxidase-positive, facultative-anaerobic and rod-shaped bacterium that is motile due to a polar flagellum. The species is considered a pathogenic aquatic bacterium, autochthonous from marine and estuarine ecosystems located in tropical, subtropical and temperate areas distributed worldwide.

The first isolation of this species occurred in 1976 when Hollis and cols. (Hollis *et al*., 1976) identified a halophilic *Vibrio* in clinical samples of blood and spinal fluid, in the USA. Those *Vibrio* isolates could ferment lactose and presented a lower tolerance for sodium chloride than *Vibrio parahaemolyticus* (Hollis *et al*., 1976). In the same year, Reichelt and cols. (Reichelt *et al.,* 1976) described the species *Beneckea vulnifica* by *in vitro* DNA/DNA hybridizations and classified the clinical isolates of Hollis *et al.* together with a collection of environmental isolates into this new species. In 1979 the species was re-assigned to the genus *Vibrio* and renamed as *V. vulnificus* (Farmer JJ 3rd, 1979).

Biotypes and serotypes. In 1975, vibrios phenotypically similar to the isolates of Hollis *et al.* but indole-negative were recovered from diseased eels (*Anguilla japonica*) in Japan (Muroga *et al*., 1976). Later, the isolates of Hollis *et al.* together with those from diseased eels were analyzed in depth by Tison and cols. who described two biotypes within *V. vulnificus*: the biotype 1 comprising the human isolates that are positive for indole production, ornithine descarboxylase activity, growth at 42ºC and acid production from mannitol and sorbitol, and the biotype 2 clustering the eel isolates that are negative for the four previously indicated tests (Tison *et al.,* 1982). According to Tison *et al*., the eel isolates were serologically identical and virulent for mice and eels. The serovar was designated serovar E by Biosca and cols. in 1996 who established a serotyping scheme based on outer membrane extraction, separation of their components by electrophoresis and

immunostaining with polyclonal antibodies against the O-antigen. (Biosca *et al.,* 1996b). The same year, an outbreak of human vibriosis among the workers of a tilapia-farm was registered in Israel. The new isolates were avirulent for eels, differed phenotypically from biotypes 1 and 2 and were grouped in a third biotype in 1999 (Bisharat *et al.,* 1999). In parallel, Amaro and Biosca reported that the biotype 2 was potentially virulent for humans after the identification of one human blood isolate from the ATCC as belonging to biotype 2 (Amaro and Biosca, 1996). From this year, the scientific community recognized that the biotype 2 was a zoonotic variant of the *V. vulnificus* species. Later, it was reported the isolation of new serovars within the biotype 2 isolated from diseased eels cultured in freshwater-eel farms in Denmark (Fouz and Amaro, 2003). The new serovars are less virulent for eels than the serovar E and were avirulent for mice (animal model used to predict virulence for humans) (Fouz *et al.,* 2010). Thus, the zoonotic variant of *V. vulnificus* is restricted to the serovar E of the biotype 2.

B.   Vibriosis

1.   Human vibriosis: modalities, risk factors and clinical signs

V. vulnificus is an opportunistic pathogen that cause a disease in humans with multiple pathologic presentations collectively called "human vibriosis". The human vibriosis can be classified in two main forms related to the disease transmission or the route of entry of the pathogen into the human body; skin contact or injuries during seawater-associated activities *versus* ingestion of raw or undercooked seafood. In both cases, the disease can lead to death by sepsis depending on a series of risk factors that are:

- Chronic liver diseases: i.e. chronic hepatitis B or C, cirrhosis due to an excess of alcohol consumption, etc.
- Immunodeficiency: i.e. due to acquired immunodeficiency syndrome, cancer or immunosuppressive chemotherapy.
- Gastrointestinal disorders.
- Diabetes mellitus.

- Renal diseases.
- Hematological disorders that cause an increase of the iron levels on serum: i.e. hemochromatosis or thalassemia.

The most dangerous of all these risk factors is the hemochromatosis; the high iron levels in serum provoke transferrin saturation levels higher than 70% (normal values are around 30%) and favor the growth of *V. vulnificus* in blood. Table 1 represents the percentage of patients with risk factors that suffered *V. vulnificus* and other *Vibrio* infections in USA (Horseman and Surani, 2011).

From the three biotypes, only the biotype 1 has been proved to infect by the oral route and cause death by primary septicemia after raw seafood consumption. The clinical signs are abdominal pain, cramps, nausea, vomiting, diarrhea, fever and chills, followed by a bacteremia produced by the invasion of the bloodstream. It is believed that the portal of entry in the bloodstream is the small intestine or the proximal colon with the ileum as the most likely site (Chen *et al.,* 2002). This primary septicemia presents a mortality rate higher than 50%, and symptoms usually occur within 7 days after infection, although they can be delayed until 14 days in some cases (Haq *et al.,* 2005).

The three biotypes are able to cause severe wound infections that can be pre-existent or be produced while fish-handling or fishing or doing some aquatic sports (Oliver J. D., 2005). In this case, the bacterium colonizes the wound (punctures, lacerations, scratches or abrasions) and causes a severe skin infection. Common clinical signs are bullae, cellulitis, ecchymosis, fever, chills, necrotizing fasciitis, necrotizing vasculitis and gangrene (Oliver J. D., 2005). Symptoms usually occur between 7 and 12 days following exposure. In patients with the mentioned underlying diseases, the three biotypes of *V. vulnificus* can invade the bloodstream and cause bacteremia that is known as secondary septicaemia (Figure 1) (Horseman and Surani, 2011).

Table 1. Percentage (%) of patients with risk factors by clinical syndrome and epidemiology study

Risk factors	Study, year (ref.)			
	Tacket *et al.*, 1984	Klontz *et al.*, 1988	Parik *et al.*, 1995	Shapiro *et al.*, 1998
Gastrointestinal		n=7		n=23
Liver disease				14
Alcoholism				14
Diabetes mellitus		14		5
Gastrointestinal disease/surgery[a]		28		11
Heart disease				10
Hematological disorder				
Immunodeficiency [b]				5
Malignancy				16
Renal disease		14		5
Any chronic disease		28		35
Primary septicemia	n=18	n=38	n=92	n=181
Liver disease		66	79	80
Alcoholism			73	65
Diabetes mellitus			4	35
Gastrointestinal disease/surgery[a]				18
Heart disease				26
Hematological disorder				18
Immunodeficiency [b]				10
Malignancy				17
Renal disease				7
Any chronic disease	89			97
Wound infection	n=9	n=17		n=189
Liver disease		12		22
Alcoholism				32
Diabetes mellitus				20
Gastrointestinal disease/surgery[a]				10
Heart disease				34
Hematological disorder				8
Immunodeficiency [b]				9
Malignancy				10
Renal disease				7
Any chronic disease	56			68

[a] Includes gastritis, pancreatitis, regional enteritis, peptic ulcer disease and ischemic bowel disease.
[b] Includes HIV: patients receiving chemotherapy or immunosuppressive drugs (including chronic corticosteroid use) for cancer, organ transplantation, rheumatoid arthritis or other autoimmune disorders; and leukopenia or neutropenia.

Figure 1. Lesions from infected wounds (A) and derivates from a secondary septicemia (B) caused by *V. vulnificus* (images from J.D. Oliver).

2. Eel vibriosis: Biology and "culture" of eels; Modalities and clinical signs; Route of transmission, portals of entry and disease process

V. vulnificus biotype 2 is the aetiological agent of the warm-water vibriosis, a disease that affects fish cultured in brackish-water (0.3-2% NaCl) at warm temperatures such as tilapia and eel (Tison *et al.,* 1982; Fouz *et al.,* 2002; Fouz *et al.,* 2007). The disease is a hemorrhagic septicaemia that is triggered irrespectively of the immune status of the fish. The model to study this kind of disease is the eel

Eels are catadromous fish that present a complex life-cycle with true metamorphoses (Tesch, 2003; Van Ginneken and Maes, 2005). The spawning area of American and European eels is the Sargasso Sea and that of Japanese eels is the

western North Pacific Ocean (Tesch, 2003; Van Ginneken and Maes, 2005). In all cases, it is believed that spawning takes place at a depth of hundreds meters. In the case of the European eel, the young larvae (leptocephali) drift towards Europe with the Gulf Stream and arrives approximately 1-3 years later (Bonhommeau *et al.,* 2010). When approaching the coast, the larvae metamorphose into the "glass eel" stage, enter estuaries and start migrating upstream. During this second migration, the glass eels metamorphose into elvers (young eels) and colonize ponds, lagoons, lakes, etc. The elver grows and turns yellow ("yellow eel" or adult eel) and, after 5–20 years, some of the eels metamorphose again, become sexually mature ("silver eels") and migrate back to the Sargasso sea to spawn (more than 6000 km against the Gulf Stream) (Tesch, 2003; Van Ginneken and Maes, 2005). The European eel (*Anguilla anguilla*) currently occupies a position in the International Union for Conservation of Nature (IUCN) red list as a critically endangered species due to multiple anthropogenic factors including habitat destruction, pollution, disease and overfishing (http://www.iucnredlist.org).

The life cycle of the eel is so complex and poorly understood that no successful methodology has been developed for its reproduction in captivity. Thus, production of eels is based on the capture of wild glass eels or elvers, and their continued growth in farms under intensive conditions (at high density in tanks) by using recirculation technology (Tesch, 2003). The physico-chemical parameters for optimal eel production are water temperature around 24 ºC, water salinity around 1% and high density in tanks (i.e. for eels weighing 50 g, densities of 100-150 kg/m^2) (Tesch, 2003). These conditions are favourable for *V. vulnificus* survival and warm-water vibriosis transmission (Marco-Noales *et al.,* 2001; Marco-Noales *et al.,* 1999). The stressing conditions in tanks (high density, handling, periodical grading…) make eels more susceptible to infectious diseases such as warm-water vibriosis.

The eel vibriosis presents two modalities depending on water salinity. The "brackish-water" modality (salinity 0.5-2%) occurs in farms as epizootics or outbreaks of high mortality and is caused by the serovar E (Biosca *et al.,* 1991; Biosca *et al.,* 1996b) (or serovar O4 according to Høi *et al.*, 1998), while the freshwater (salinity 0,3-0,9%) modality occurs as outbreaks of low mortality and is caused by serovars A and I (Fouz *et al.,* 2006; unpublished results) (serovar I is

equivalent to serovars O3 and O3/O4, according to Høi et al. [Høi et al., 1998]). In both cases, the external lesions appear first as petechiae on the abdomen, hemorrhaging of the anal fin and a reddening in the opercular region (Figure 2). Protrusion of the rectum is also sometimes observed. The anterior part of the belly is often swollen and the skin shows pathological changes which sometimes progress to large ulcers (2-4 cm in diameter) with central whitish-yellow necrotic tissue (Figure 2). Some ulcers can develop small perforations in the center. Common internal signs are inflammation of tissues and the intestinal canal, pale and hemorrhagic liver, swollen kidney and purulent ascitic fluid in the abdominal cavity. The specific clinical signs are ulcers on the head, in the case of the brackish water modality, and jaw degradation in the case of the freshwater modality (Figure 2).
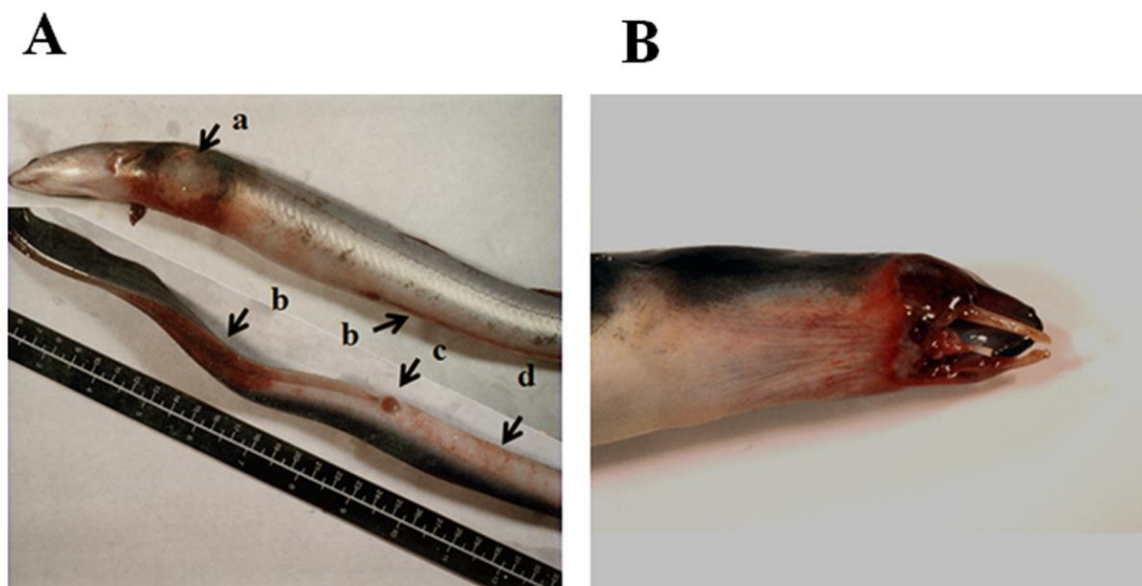
Figure 2. **Clinical signs of warm water vibriosis in eels**. A) Eels affected by vibriosis caused by serovar E with the typical clinic signs (hemorrages [b], protruding annus [c] and petequias [d]) as well as the specific of serovar E (ulcers [a]); B) eel showing the specific clinical sign of the serovar A (raw degradation) (image from Biosca et al., 1991).

Water is the prime vehicle for the transmission of both modalities of vibriosis, serovar E adapted to be optimally transmitted in "brackish-water" and serovars A and I in "fresh-water". In addition, serovar E and non-serovar E strains use different portals of entry into the eel's body: the gills, in the case of serovar E, and the anus, in the case of non-serovar E, which correlates with the hemorrhagic faeces observed in the tanks during the outbreaks caused by non-serovar E strains (Marco-Noales *et al.,* 2001; Fouz *et al.,* 2010). Examination of the survivors by indirect immunofluorescence and scanning electron microscopy shows that *V. vulnificus* biotype 2 forms a biofilm-like structure on the eel's skin surface (Marco-Noales *et al.,* 2001) (Figure 3).

It has been a matter of speculation if *V. vulnificus* also infects eels in nature. The most accepted hypothesis is that eels become infected with *V. vulnificus* in the coastal waters, mainly estuarine waters, and carry *V. vulnificus* to the aquaculture installations when they are captured. In fact, some mild warm-water vibriosis cases among wild-eels in lakes have been reported. These cases were produced by non-serovar E strains (Amaro *et al.,* 1995).

Eel colonization and invasion experiments performed with serovar E demonstrated that this serovar multiplies on the gills following saturation dynamics, subsequently invades the blood stream by an unknown mechanism and spreads to the internal organs where it reaches population sizes that are notably lower than those associated with other vibriosis (Valiente *et al*, 2006; Valiente *et al*, 2008a).
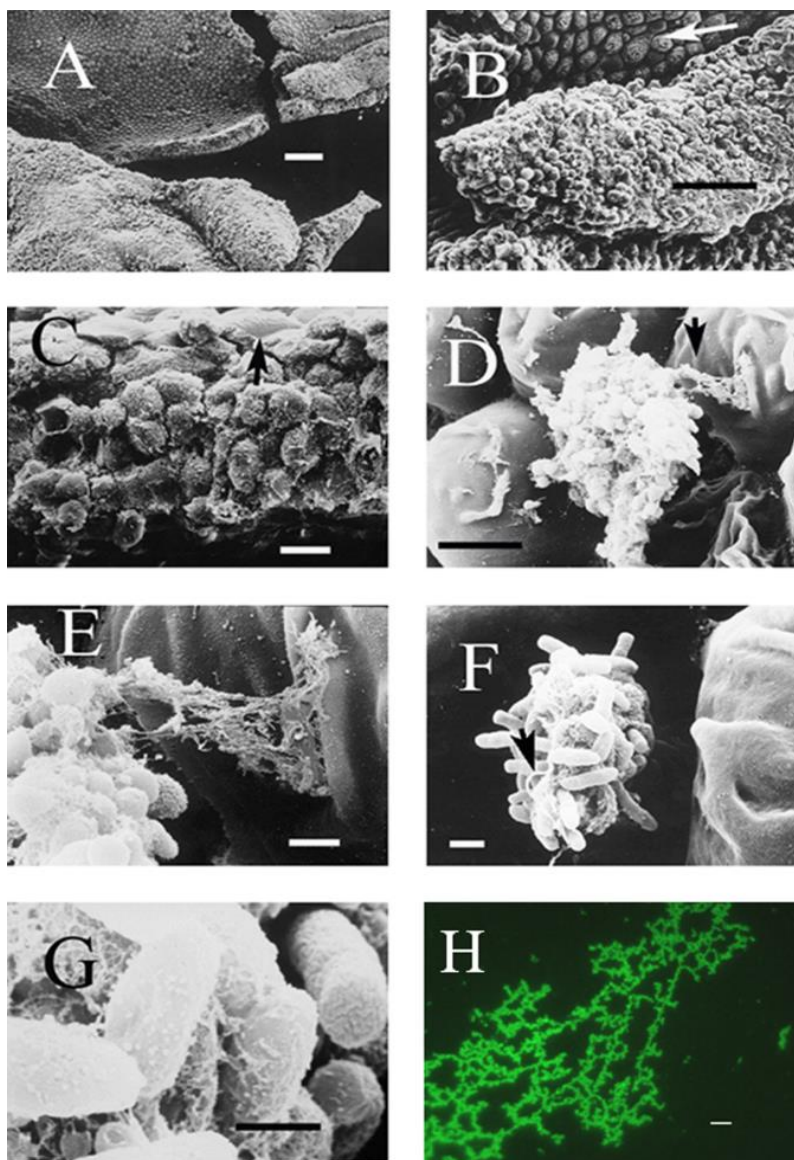
Figure 3. **Images from Scanning Electron Microscope of the eel surface.** Samples correspond to the covering that presented some dead eels by a *V. vulnificus* serovar E infection, after 15 days in which cohabitation between healthy and disease fishes was studied. In A, B and C can be observed that the covering is the eel skin, concretely the epidermis and the dermis. In B, both strata are randomly positioned, as consequence of sample preparation and in C can be observed the typical structure of the skin; in both images, epidermis is marked with an arrow. Groups of bacteria were found (D-G) adhered to the epidermis by an extracellular substance that also covered bacteria (D-G). In D is marked with an arrow a part of the image enlarged in image E. In F the arrow marks a bacterial flagella. In H, *V. vulnificus* serovar E in the surface of an infected eel visualized by a micrography of epifluorescence by using an antiserum anti-*V. vulnificus*. Bars represent 100 μm (A), 50 μm (B), 10 μm (C), 5 μm (D), 1 μm (E and F), 0.5 μm (G) or 2 μm (H). Image from Marco-Noales *et al.* (2001).

Parallel to bacterial spreading, extensive hemorrhages are produced in all the organs and the animal dies in less than 72 h (Valiente *et al.,* 2008a). The observation with light and electronic microscopy of the internal organs of diseased animals shows that hemorrhages are the only evident alteration. Although hemolysis is not obvious, non-specific changes, such as a slight alteration of the mitochondria structure in the hematopoietic cells of head kidney, and mildly increased number of phagocytosed erythrocytes in the spleen at 24 h and 48 h after challenge, are observed (Figure 4). Finally, the granulocytes are the main cell type that show clear signs of damage, which is evidenced by the release of cytoplasmic content, including granules (Figure 4). Either none, or very few bacteria are observed in the tissues of infected eels, and they are mostly close or within the lumen of capillary vessels (Figure 4). The rapid death of eels without gross clinical signs after infection by serovar E is congruent with studies that suggest that the eels die from a peracute septic shock.
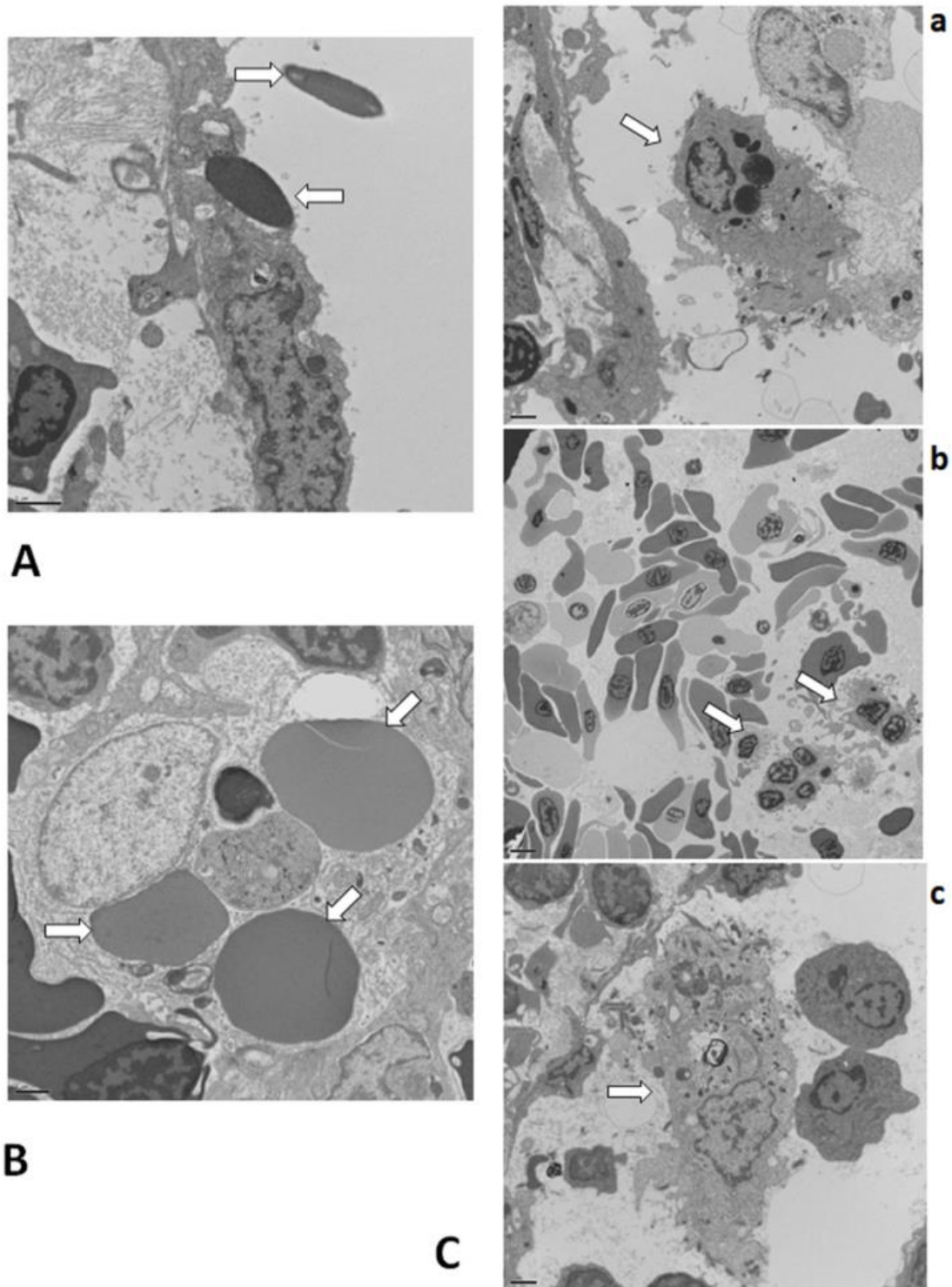
Figure 4. **Histological analysis of the eels infected with the wild-type strain CECT4999**. A) Two bacteria (marked with arrows) in a renal capillary. Notice that one of them is closely associated to an endoteliocyte. Bar, 1 μm B) macrophage with damaged erythrocytes (marked with arrows) engulfed within its cytoplasm. Bar, 1 μm. C) Three images of headkidney showing damaged granulocytes (marked with arrows): a) bar, 1 μm; b) bar, 5 μm, and c) bar, 2 μm. Image from Lee *et al.* (2012).

C.   Epidemiology, habitat and reservoirs

V. vulnificus (in fact, the biotype 1) inhabits water as free living form or associated to the mucous surface of aquatic animals, algae and plankton (Oliver, 2006). Filter organisms, such as oysters, clams and mussels accumulate this microorganism especially in warmer months and are considered as the main reservoir of this human pathogenic biotype. Temperature and salinity are the two major factors that determine the distribution of *V. vulnificus* biotype 1 in the aquatic environment. Thus, this biotype can be recovered in culturable form from water with a sanity between 4 and 37 $^0/_{00}$, with an optimum at 10-25 $^0/_{00}$, and at a range of temperatures between 7 and 36ºC with an optimum around 20 ºC (Motes, 1998). Out of these values of temperature and salinity, it is hypothesized that *V. vulnificus* biotype 1 can survive but in a viable but non culturable state (i.e. at temperatures between 0 and 4ºC and salinities between 0 and 4 $^0/_{00}$) (Oliver, 1995).

An important point that influences the geographical distribution of *V. vulnificus* biotype 1 and that of most of the marine bacteria is the global climate change. Global changes in temperature, even only the small ones, affect the biology of marine bacteria and therefore their geographical distribution and abundance. In fact, an increment of 5ºC has been shown to play a significant role in the increase of incidence of *Vibrio* infections (Baker-Austin *et al.,* 2010). In the last decades changes in water temperature has been observed in Southern Europe and in the Black Sea region where the increase has been around 4-5 ºC, and in Western Europe with an increase of 2.5 – 3.5 ºC (Baker-Austin *et al.,* 2010). These alterations extend the areas in which a marine pathogen like *V. vulnificus* biotype 1 can be present and therefore increase the risk of contact and transmission to humans. In fact, about 85% of human vibriosis occur between May and October, when the water temperature is closer to the optimal (18-20ºC) (Baker-Austin *et al.,* 2010). Similarly, low-level flooding associated with sea-level change leads to a decrease of the salinity of some areas and potentially expands the geographic distribution of *V. vulnificus* biotype 1, as well as other natural phenomena that produce the same effect, like hurricane Katrina in August 2005, since this *Vibrio* prefers low salinity levels (Baker-Austin *et al.,* 2010).

Although human vibriosis infections are relatively rare, *V. vulnificus* biotype 1 is one of the food-borne pathogens that has shown a great increase in its incidence in the last years. In fact, recent data estimates that between 1996 and 2005 the annual incidence of *V. vulnificus* biotype 1 has increase 41% (Baker-Austin *et al.,* 2010). There are several possible explanations for these data, i.e. the increase in consumption of seafood in the last years that is much higher than in the early 1970s, thus expanding the contact of *V. vulnificus* biotype 1 to humans with risk factors. On the other hand, there has been an increase of individuals with pre-disposing risk factors in the global population due to an expansion of HIV that alter the immune system and the longer expectancy of life of people with liver chronic diseases or serum iron disorders. In Europe and surrounding areas, cases of *V. vulnificus* infections (regardless the biotype) have been detected in Israel, Denmark, Turkey, Germany, Sweden, Spain, Greece and Belgium, as it can be seen in Figure 5. In most of the cases, there is a clear relationship with water temperature, since the infections were registered in the warmest months (Baker-Austin *et al.,* 2010).

V. vulnificus biotype 2 emerged in Japan in 1976 and arrived to Europe with carrier eels in the early 80´s. The European and Japanese isolates belonged to serovar E. These first isolates differed from biotype 1 strains in a few biochemical tests (see above) and in host specificity. The serovar E spread from Spain to Nordic countries and caused important economic losses in multiple eel farms mainly located in Spain, Germany, Holland and Denmark.

In the mid 80's, fish farms decided to change brackish water by freshwater to control the severity of vibriosis outbreaks in spite of the reduction in market-eel production. This measure was good since the incidence of outbreaks of eel vibriosis reduced significantly. However, the measure propitiated the emergence of new serological variants of biotype 2 better adapted to the new salinity conditions. The new serovars, called serovars A and I, differed from serovar E in that they were biochemically similar to biotype 1 isolates, less virulent to eels and avirulent to humans (the mouse was used as animal model). Serovars A and I emerged at the end of 80's in Nordic countries and arrived to Spain in 2000.

Figure 5. **Selection of reported infections of *V. vulnificus* in Europe, indicating geographical spread of reported cases**.

V. vulnificus biotype 2 was considered to be an obligate eel pathogen whose main reservoir was the farmed-eel. Marco-Noales and cols. performed a series of experiments of survival in natural and artificial microscosms to demonstrate that this biotype, and in particular, the zoonotic serotype, is also part of the natural aquatic microbiota (Marco-Noales *et al.,* 1999). The authors showed that the zoonotic variant survived in artificial water microcosms under starvation for years either associated (biofilm) to hydrophilic and hydrophobic surfaces or as a free living form (Marco-Noales *et al.,* 1999). They also demonstrated that the survival of the zoonotic variant in natural water microcosms was mainly controlled by biotic factors such as competence with other bacteria (including the biotype 1 of the species) and predation by protozoa. In any kind of microcosms, the survival of the

biotype 2-serovar E was controlled by water salinity and temperature as it occurs with the biotype 1 of the species. Later, Sanjuán and Amaro developed a two-step protocol for its specific isolation from aquatic samples that uses saline eel plasma instead of alkaline peptone water as enrichment broth (Sanjuán and Amaro, 2004). The protocol allowed the isolation of this biotype and serovar from water samples and healthy fish demonstrating that the zoonotic variant is part of the natural aquatic microbiota.

In 1996, it was reported that serovar E isolates were potentially virulent for humans after the identification of one human blood isolate from the ATCC as belonging to biotype 2 and serovar E. This isolate came from a septicemic case registered at the USA. After this report, a few additional human isolates from Germany, Holland, Sweden, France and Australia (a country where eels are not cultivated) were also identified as belonging to biotype 2 and serovar E, confirming that this serovar is a zoonotic variant that constitutes a potential risk of infection. The correctly traced human isolates of biotype 2 came from necrotic wounds and secondary septicemia cases after wild or cultured fish manipulation. These epidemiological data lead us to conclude that the zoonotic variant can infect sporadically human wounds after fish manipulation that can produce a secondary septicemia if the patient belongs to a group of risk.

D.   Phylogeny

To find out the evolutionary history of *V. vulnificus*, sequence-based analyses of housekeeping genes was performed by Cohen and cols. (Cohen *et al.,* 2007). The phylogenetic tree was generated on basis on variability of sequences of six housekeeping genes and divided the species into two lineages that they called Clinical and Environmental, since they were apparently related to human pathogenic potential. The Clinical lineage grouped most of the biotype 1 isolates from human clinical cases and was more diverse than the Environmental one, which clustered most of the environmental biotype 1 isolates including all the analysed biotype 2 strains, all of them of serovar E and isolated from diseased eels. The strains

of biotype 3, as well as two strains of biotype 1, did not cluster with none of these lineages. Later, Sanjuán and cols. (Sanjuán *et al,* 2011) performed a similar study with the objective of clarifying the phylogeny of the biotype 2. To this end, the authors used more than 100 strains of the three biotypes, including biotype 2 strains belonging to the three serovars from both clinical (from diseased humans and animals) and environmental origin (water, shrimps and fish). The authors sequenced and analysed the variability of three virulence-associated genes and four housekeeping ones and performed a multilocus sequence analysis. They found that *V. vulnificus* species had a mixed population structure formed by multiple single sequence types from different origins together with clones and clonal complexes, all of them from fish-farm-related environments. The concatenated phylogenetic tree clearly shows that the species is divided in three lineages (Sanjuán *et al,* 2011) (Figure 6). LI, corresponding to the predefined Environmental lineage of Cohen and cols. (Cohen *et al.,* 2007), groups biotype 1 and 2 strains from environmental samples mostly related to fish farms, biotype 2 strains from diseased animals and biotypes 1 and 2 strains from human cases of wound infections and secondary septicaemia. LII cluster all the biotype 3 isolates, which constitute a clon (Figure 6). Finally, LIII, corresponding to the previously described Clinical lineage by Cohen and cols. (Cohen *et al.,* 2007), comprises biotype 1 isolates from the seawater and seafood but not cultured fish, and human isolates from blood (most of them from primary septicaemia) (Figure 6). Interestingly, biotype 2 isolates appeared in LI distributed within different subgroups related to the serovar, and in each subgroup, they were closer to biotype 1 isolates from fish-farms than to each other (Figure 6). These results strongly suggest that acquisition of new information by horizontal gene transfer (HGT) and further recombination could have given rise to the emergence of the so-called biotype 2. In fact, the authors found evidences of recombination affecting some of the studied genes in the biotype 2 strains. Finally, the authors suggested that the polyphyletic origin of so-called biotype 2 would support its reclassification within the species as a pathovar (pv. *piscis*) that would group the strains with pathogenic potential to infect and develop vibriosis in fish. The rest of the strains of the species should be classified in genogroups with different phylogenetic origin and potential to infect humans. This proposal needs more experimental support to be accepted by the scientific community.

The phylogenetic study of Sanjuán and cols. also evidenced that the human isolates of *V. vulnificus* of the three biotypes could be separated from the others on the basis of variations in the *pilF* gene sequence. Later, Roig and cols. (Roig *et al.,* 2010) demonstrated that *pilF* is a good genetic marker for human virulence potential in this species because it is correlated to human plasma resistance. The same authors developed a multiplex PCR methodology to identify the strains potentially dangerous for public health and, later, Baker-Austin and cols. (Baker-Austin *et al.,* 2012) a real-time PCR assay for their detection from oysters. PilF is a protein required for pilus type IV assembly, whose mutation in other bacterial pathogens is involved in attenuated virulence for mice (Chakraborty *et al.,* 2008). The exact role of *pilF* in virulence for mice/humans in *V. vulnificus* species has yet to be determined.

0.05
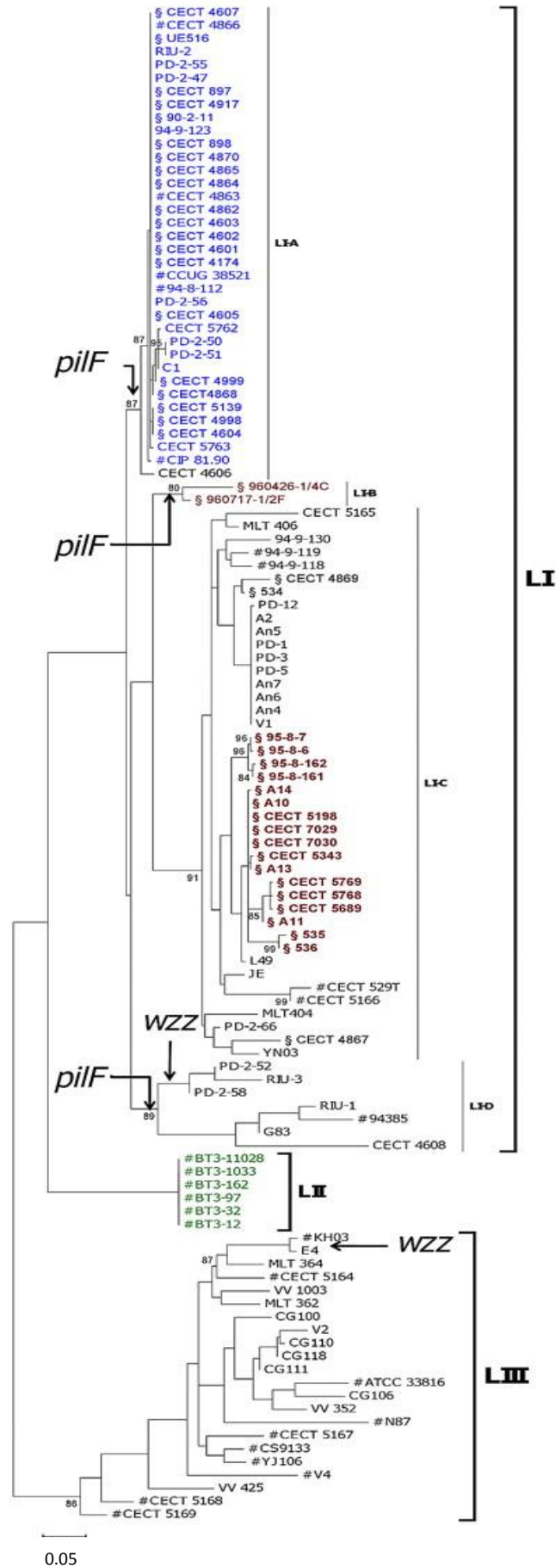
Figure 6. **Maximum likelihood phylogenetic tree of 115 *V. vulnificus* isolates obtained from the alignment of 7 concatenated loci.** Black, biotype 1 isolates; blue, biotype 2 serovar E isolates; red, biotype 2 non-serovar E isolates; green, biotype 3 isolates; #, human isolates; §, diseased fish isolates. Branches where recombination events involving the indicated loci might have occurred are indicated by arrows. The numbers at the nodes represent the percentage values given by bootstrap analysis of 1.000 replicates. Image from Sanjuán *et al.* (2011).

E. Genomes and plasmids

Genomes

The first genome of *V. vulnificus* was sequenced and published in 2003 (Chen *et al.,* 2003). The sequenced strain, called YJ016, is a biotype 1 strain isolated from blood of a septicemic patient in Taiwan. Its genome contains a large and a small chromosome together with a conjugal plasmid, pYJ016, with a total of 5.028 ORFs (Open Reading Frames), including virulence genes such as those for cytolysins, proteases, and capsular polysaccharide biosynthesis as well as iron-uptake systems. In the same year, Kim and cols. (Kim *et al.,* 2003a) published the genome of another biotype 1 strain also from blood of a septicemic patient, the strain CMCP6, in this case isolated in South Korea. This strain was re-sequenced and the genome was re-annotated years later by the same research group (Kim *et al.,* 2011). The genome is also formed by two chromosomes of similar size to those of the strain YJ016 and also presents a similar G+C content. The authors identified some genomic islands probably acquired by horizontal gene transfer events that confirmed the findings of Quirke and cols. (Quirke *et al*. 2006) obtained after amplification and sequencing of specific zones by using primers derived from YJ016 and CMCP6 strains. These authors hypothesized that these horizontal gene transfer (HGT) phenomena would have been the responsible ones for the diversification of each genome, leading to an increase on fitness of this species under varying environmental conditions (Quirke *et al*. 2006). The main features of the genome of the strain YJ016 are summarized in Table 2.

Since then, many *V. vulnificus* strains have been sequenced by diverse research groups; MO6-24/O (Park *et al.,* 2011), B2 (Wang *et al.,* 2012), E64MW (Morrison *et al.,* 2012), JY1305 (Morrison *et al.,* 2012), JY1701 (Morrison *et al.,* 2012), VVyb1 (Danin-Poleg *et al.,* 2013) and ATCC27562 (Li *et al.,* 2012), and other genome projects are in progress. One of these projects is the genome of one strain belonging to the zoonotic variant, the strain CECT4999. This strain was isolated in 1999 from a diseased European eel.

The main features of the genome of strain CECT4999 in comparison of that of strain YJ016 are summarized in Table 2. The zoonotic strain has two chromosomes and a plasmid higher than pYJ016 with a total of 4.533 coding DNA sequences or ORF. An 82% (3.698) of ORF show significant homology to previously identified genes and the rest corresponds to hypothetical proteins. The genome sizes of the two strains are comparable and both harbor a large super-integron of 195 kb on the chromosome 1 (Table 2).

Table 2. Global features of the *V. vulnificus* genomes (unpublished data).

	Location	YJ016 (Biotype 1)	CECT4999 (Biotype 2)
Size (bp)	**ChrI**	3.354.505	3.394.464
	ChrII	1.857.073	1.700.225
	Plasmid	48.508	68.446
GC percentage (%)	**ChrI**	46.4	46.3
	ChrII	47.2	47.1
	Plasmid	44.9	43.8
Total number of ORFs	**ChrI**	3.262	3.030
	ChrII	1.697	1.432
	Plasmid	69	71
Number of tRNAs	**ChrI**	100	103
	ChrII	12	15
	Plasmid	0	0
Number of rRNA operons	**ChrI**	8 (25 ORFs)	8 (25 ORFs)
	ChrII	1 (3 ORFs)	1 (3 ORFs)
	Plasmid	0	0
Super-Integron	**Chr1**	1.791.850 – 1.930.850 (138 kb)	1.371.914 – 1.567. 221 (195 kb)

The distribution of proteins in different Clusters of Orthologous Groups (COGs) was also similar, except for the carbohydrate transport/metabolism [G] and replication/recombination/repair [L], which were more abundant in YJ016 and CECT4999, respectively (Figure 7). Overall the profile of the two biotypes of *V. vulnificus* is very similar and differs only slightly from that of *Vibrio anguillarum* 775, presented for comparative purpouses. The distribution of COGs on the two chromosomes is also similar in all the three genomes with chromosome 1 encoding a majority of housekeeping and other essential genes and chromosome 2 encoding genes related to virulence and adaptations to the respective environments.
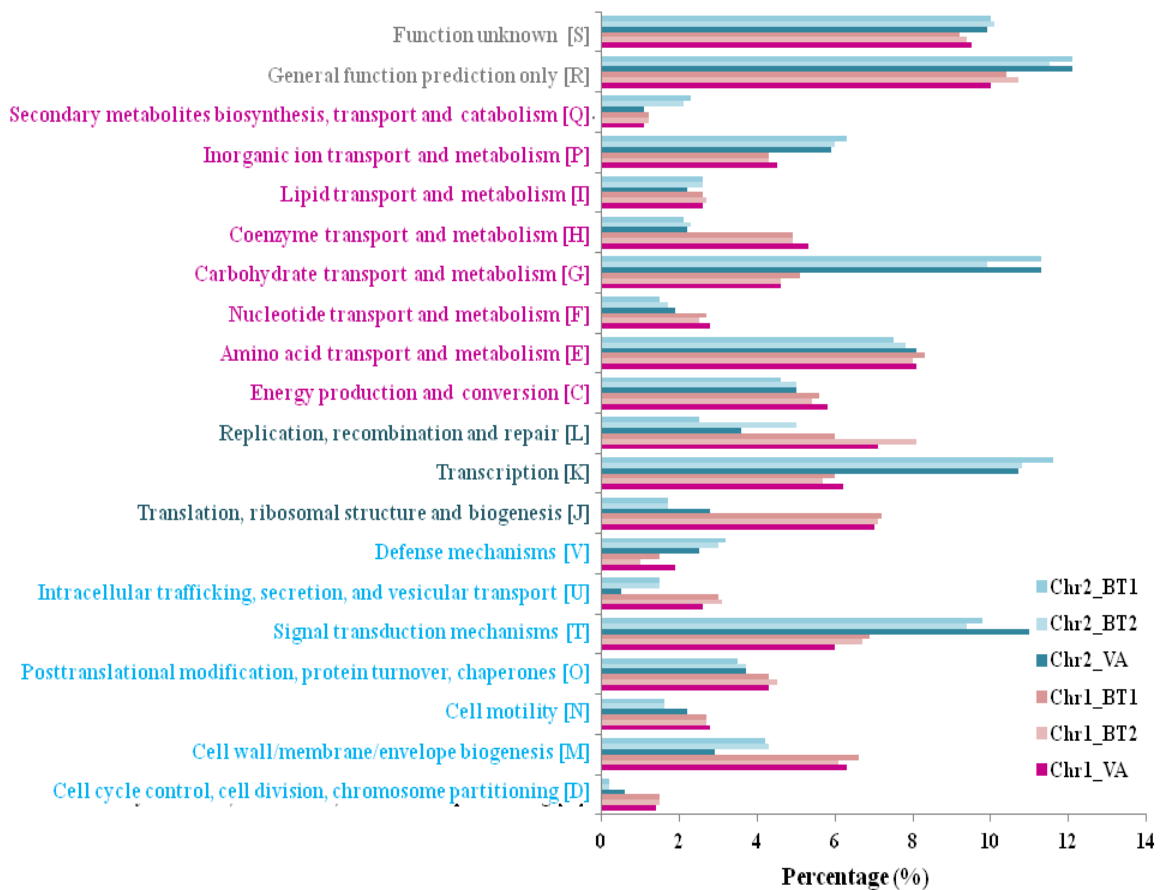
Figure 7. Percentage COGs distribution of predicted ORF in the *V. vulnificus* strain CECT4999 (BT2), strain YJ016 (BT1), and fish pathogenic *Vibrio anguillarum* 775 (VA) (unpublished data).

Out of 469 ORF uniquely present in CECT4999, 236 (50%) were annotated as hypothetical proteins and 94 (20%) were annotated as transposases and other mobile elements. Among the remaining 30% of the genes, are those involved in carbohydrate transport and metabolism, cell wall biogenesis, the *dnd* cluster, restriction and modification related methyltransferases and endonucleases, transcriptional regulators, chemotaxis related and histidine kinases involved in signal transduction, and toxin-antitoxin related are a few most prevalent ones. 60 biotype 2 unique ORF were predicted as putative extracellular proteins of which 42 (70%) were hypothetical in nature. An N-terminal signal peptide cleaved by signal peptidase I were found in 24 biotype 2 unique ORF confirming their secretory nature including a transthyretin like protein and a transferrin-binding protein A precursor encoded by the plasmid pR99 (described later). Other 10 proteins were predicted as lipoproteins based on the presence of an N-terminal signal peptide for the signal peptidase II, including the known eel virulence factor 'RTX toxin and related Ca2+-binding protein' encoded by the plasmid pR99. Interestingly, most of these ORF are located in mobile genetic elements such as genomic island and plasmids, corroborating the hypothesis of Quirke and cols. (Quirke *et al.* 2006) about the role of genetic mobile elements in adaptation to specific niche or environment. Special importance deserves the plasmid pR99 identified in the genome of the zoonotic strain, which will be described in depth.

Plasmids

In 2005, Lee and cols. (Lee *et al.,* 2005) hypothesized that the virulence determinants for eels in *V. vulnificus* biotype 2 should reside in the DNA regions that are common to all biotype 2 strains, but are absent from biotypes 1 and 3. To identify these genetic regions, the authors compared the whole genome of one biotype 2-serovar E strain with those of three biotype 1 strains by Suppression Subtractive Hybridization (SSH). The authors identified eight sequences, of which only three were present in all biotype 2 strains, regardless of its serovar, and absent in the rest of the biotypes. The three specific-biotype 2 sequences were plasmid-borne, which suggested the involvement of plasmids in virulence for eels. Related to this

discovery, Biosca and cols. (Biosca *et al.,* 1996b) and later Lewin and cols. (Lewin *et al.,* 2000) had previously reported that biotype 2-serovar E isolates carried a plasmid of 68-70 kb and that this plasmid was strongly conserved among strains since restriction length polymorphism and hybridization analysis did not reveal substantial differences. Lee and cols. (Lee *et al.,* 2008a) sequenced the plasmid of the CECT4999 strain, named pR99. The main features of the pR99 plasmid are shown in Figure 8.
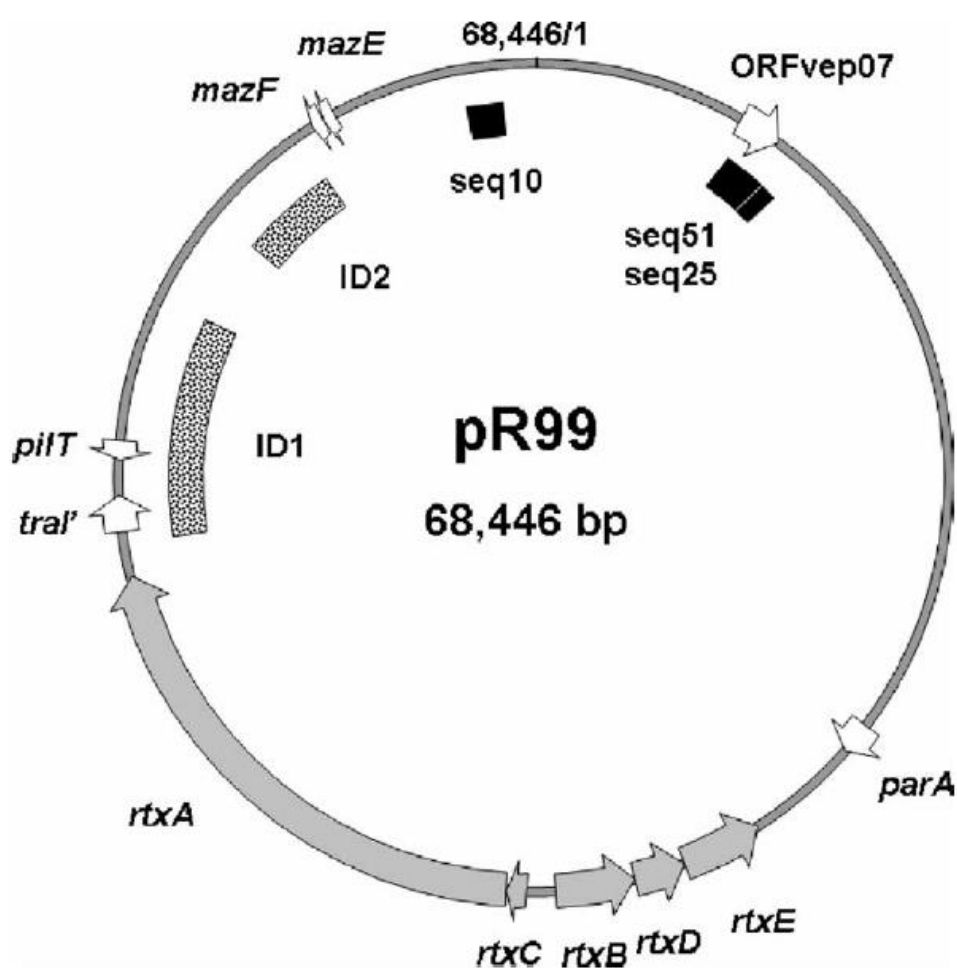
Figure 8. **Main features of the plasmid pR99 in *V. vulnificus* strain CECT4999**. Some of the predicted ORFs are indicated with arrows. The ORFs associated with production of an RTX toxin, conjugative transfer of plasmids, and other functions are indicated in gray, white and black, respectively. Regions ID1 and ID2 are indicated with stippled bars. Image from Lee *et al.* (2008a).

Plasmid pR99 had a size of 68.5 kb and show a G+C content of 43.3%, containing 71 predicted open-reading frames (ORFs) (Figure 8). Plasmid pR99 contained genes encoding RTX (repeats in toxin) toxin and those related with transposition and other known and unknown functions (Figure 8) and presented the operon *mazEF* encoding a toxin-antitoxin system involved in maintenance of plasmids. Intriguingly, the pR99 plasmid presented two homologous zones designated as ID1 and ID2, whose function will be described later (Figure 8). The presence of transposons together with the high similarity to other genes present in plasmids from other aquatic bacteria suggests that this plasmid, like many other plasmids, is a mosaic composed of regions from multiple sources that might have been formed via horizontal gene transfer between aquatic bacteria.

To demonstrate that pR99 was a virulence plasmid, Lee and cols. (Lee *et al.,* 2008a) obtained a pR99-cured strain from CECT4999, and tested it for eel virulence and resistance to eel plasma. To obtain the cured strain, it was necessary to inactivate the operon *mazEF,* involved in the maintenance of the plasmid in *V. vulnificus*. The cured strain was completely avirulent for eel and sensitive to fresh eel plasma (Lee *et al.,* 2008a; Valiente *et al.,* 2008c). The introduction of pC4602-2 plasmid, 92% identical in nucleotide sequence to pR99, into the cured strain restored the virulence and the ability to grow in fresh eel plasma, demonstrating that both pR99 and pC4602-2 are virulence plasmids (generally called pVvbt2 to appoint the virulence plasmid for all biotype 2 strains) (Lee *et al.,* 2008a). Thus, pVvbt2 encodes a system of resistance to the bactericidal activity of eel plasma that allows the bacterium to invade and spread to the internal organs of the eel. None of the annotated ORFs show significant homology with known genes related to resistance to plasma killing, *vep07* and *vep20* are the only identified genes that are putatively involved in resistance to eel plasma (see later).

To find out whether pVvbt2 also plays a role in the initial steps of infection, the wild-type strain and the cured strain were used in colonization experiments (Valiente *et al.,* 2008c). The results demonstrated that this plasmid does not play a significant role in surface colonization, since both strains were equally chemoattracted towards and adherent to eel mucus and gills. In addition, the cured strain persisted in the gills of bath-infected eels for weeks post-infection (Valiente

et al., 2008c). The virulence plasmid was later found in all the analysed biotype 2 strains and its high homology was confirmed by hybridization with specific probes (Roig and Amaro, 2009).

Interestingly, loss of virulence plasmid does not affect either the virulence in mice or growth in human blood, which suggests that encodes for host-specific virulence system (Lee *et al.,* 2008a). To find out if this host specificity could be extended to other fish species, the virulence and resistance to plasma from sea bass (*Dicentrarchus labrax*) of the cured and the wild-type strains was determined. The cured strain showed attenuated virulence and a significant reduction in resistance to sea bass plasma, suggesting that this plasmid codes for resistance to a general bactericidal mechanism developed by teleosts (unpublished data). The identification of such a mechanism would be crucial for a better understanding of both the pathogenesis of warm-water vibriosis and the innate immunity in teleosts.

According to the hypothesis based on the chromosomal phylogenetic study of Sanjuán *et al.* (Sanjuán *et al.,* 2011), biotype 2 is polyphyletic and would have emerged by the acquisition of new genetic information by horizontal gene transfer in the nutrient-enriched fish farming environment. Lee and cols. (Lee *et al.,* 2008a) identified the genetic element as a virulence plasmid and the transference mechanism as conjugation facilitated by a conjugative plasmid. The acquisition of pVvbt2 could have occurred before or after the divergence of the common ancestor for biotype 2 strains. In the first case, avirulent fish isolates that are close to biotype 2 isolates in the phylogenetic tree would have lost the virulence plasmid. In the second case, the process of plasmid acquisition would have been produced several times, favored by the nutrient-enriched environment of fish-farms. The second possibility seems to be less plausible since the virulence plasmid seems to be highly conserved, as little or no genetic variation has been observed in the sequences of some genes from a selection of biotype 2 strains belonging to three different serovars (Lee *et al.,* 2008a; Roig and Amaro, 2009; unpublished results). Nevertheless, to clarify the origin of the so-called biotype 2, more plasmids should be sequenced and their phylogeny constructed and compared to that of the chromosomes.

II.   Animal models to test virulence of *V. vulnificus*

The use of animal models in the study of infectious diseases is considered as an essential tool for discovering their causes, treatment and prevention as well as to find out the role of bacterial specific factors or genes in virulence. Ideally, an animal selected as the model to study human or fish infections should acquire the disease by the natural route and manifest the same clinical signs than the natural disease.

To perform these experiments is required to get a permit from an Institutional Ethical Committee. The essential question is; does the model involve causing significant pain to the animal? To minimize unnecessary animal pain and suffer, the researcher has to justify the necessity of the animal experiments. For this reason, it is desirable that animal experiments should be designed to test critical hypothesis that can provide useful information for understanding or controlling the bacterial disease studied; i.e. the role of a gene in virulence. The protocol to be approved should explain why the proposed model was chosen, ways to minimize the number of animals required and obtain statistically significant results, precedent of the use of the model by the scientific community and documentation of appropriate training by the laboratory personal. All the protocols with animals used in the present work were approved by the Ethical Committee of the University of Valencia.

Two are the animal models used to study the septicemic variant of the human and fish vibriosis caused by *V. vulnificus*; the first one, the European eel (*Anguilla anguilla*), the main natural host for the warm-water vibriosis, and the second one, the mouse, the most commonly animal model used to emulate the human vibriosis. Both animal models are infected by different routes (see later) with serial ten-fold dilutions of a known bacterial inoculum, the course of the infection is followed for 1-2 weeks, and death is recorded only if the bacterium is recovered in pure culture from internal organs of the moribund animals. Then, the virulence degree is calculated as fifty lethal dose (LD_{50}) by applying the formulation of Reed and Münch (Reed and Münch, 1938). In the colonization and invasion experiments, external and internal organs are sampled at different time intervals and the number of bacteria

per gram of organ is expressed as colony forming units on general agar media. Usually, the number of CFU per gram of organs is plotted in a log. scale and the graphs corresponding to the wild-type strain and their mutants or to different isolates compared by using different statistics to test significance.

A. Eels

Two types of experiments are performed with eels; virulence degree determination and colonization/invasion assays. For both assays adult non-immunized farmed eels are maintained in tanks containing brackish water (1.5 % of salt) at 25ºC with constant aeration. In both assays, eels can be infected by immersion, orally, intraperitoneally or anally. The intraperitoneal route is the preferred one to compare the virulence degree of different strains or that of the wild-type strain with their mutants. The bacteria are injected (0.1 ml of a known dilution) with a syringe directly in the peritoneal cavity. By this route, bacteria are able to access the bloodstream and the internal organs provoking the death of the host in less than 72 h (the experiment last 1 week).

The second route of eel infection that is commonly used is the immersion challenge. This is the natural route of warm-water vibriosis transmission caused by the zoonotic strains, and for this reason, the conclusions of the experiments can be translated directly to the natural disease. Basically, it consists in place the eels in a solution of bacteria at a given concentration for a determined time (generally 1 hour), and then, infected fishes are placed in common storage conditions recording deaths. Contrary to the i.p. injection, the death of the fishes is commonly slower and the experiment lasts 1 week. Although theoretically, death by this route of infection can be influenced by external factors, such as starvation, storage stress or interaction with other fishes, and it is not possible to know the exact inoculum of bacteria that enters in blood, this infection model is quite repetitive and reproducible. This route of infection is used for the colonization and invasion experiments whose objective is to determine the exact role of a gene in virulence; i.e. either involved in colonization or in invasion or in tissue/cell destruction. To this end, eels are bath infected and a sample from organs is taken from a pool of three

fishes (i.e. from liver, brain, blood, kidney, spleen...) at different time intervals and processed for bacterial counts on general media.

B.   Mice

Mice are the commonly accepted model to study human virulence of *V. vulnificus*. These mammals are easy to handle, its breeding can be controlled without apparent difficulties and the results are highly reproducible. In addition, the researchers can buy specific strains of genetically identical mice, which minimize the differences among experiments. Two kinds of general murine models of infection are used regardless the infection route, the normal one and the iron-overloaded model. This last model relates a decrease in LD_{50} with an increase of iron in blood, the most important risk for death among humans infected with *V. vulnificus*. Briefly, it consist in an intraperitoneal (i.p.) injection of a source of non-toxic iron two hours before the experiment. The most common iron sources are hemoglobin, hemin, and ferric ammonium citrate.

The i.p. injection in mice is the route of choice in case of the septicemic variant of the vibriosis caused by *V. vulnificus*. The procedure, the time to death and the time period of the experiment are the same than the used with eels differing in the volume of bacterial dilution inoculated per mouse, 0.2 ml. The intradermical injection is another route of infection where the bacterial inoculum is applied directly in the dermis. In this route the dose takes more time to take effect, and is used generally to emulate an infected open wound, one of the ways of entry of *V. vulnificus*, where the dermis is exposed and in contact with the pathogen. Although this route can be used by the zoonotic serovar to infect humans, the results obtained by different laboratories are not reproducible, which advises against its use. Finally, another way by which a patient can be infected with *V. vulnificus* is through ingestion of contaminated seafood. *V. vulnificus* is considered one of the most important emerging foodborne pathogens, and details of the vibriosis infection through the oral-contamination are interesting points to be studied. To this end, the model of infant mouse intragastric model is the most currently used. In brief, it consists in applying a bacterial inoculum directly into the stomach of the mouse with a syringe

and assesses different parameters like virulence degree. This animal model is particularly useful when studying, i.e., virulence factors that are involved in destruction of intestinal epithelium and invasion of the bloodstream, but it is only applied to biotype 1 strains.

III. Virulence factors

Most of the virulence studies on *V. vulnificus* have been performed with biotype 1 strains and have been focused on human virulence by using the mouse as animal model. Assuming that no plasmid is present in the majority of the sequenced biotype 1 strains from clinical origin, it can be concluded that the human virulence genes are located in one or the two chromosomes and the fish virulence genes are located both in the plasmid and in the chromosomes.

To better understand how warm-water vibriosis of biotype 2 occurs, the disease can be divided into three temporal phases: gill colonization (colonization phase), blood invasion and spreading to the internal organs (invasion phase), and, finally death by a hemorrhagic septic shock (lesional phase). Since all the work has been performed with the zoonotic serovar, the putative role of each virulence factor in human vibriosis will be described and the differences between the two hosts (teleosts and mammals) commented on.

A.   Colonization and invasion factors

1.   Appendages: flagellum and pili.

The flagellum is a locomotive organelle that confers bacteria the ability to swim in liquid and swarm on solid surfaces contributing substantially to the adhesion, biofilm formation and invasion of host cells and tissues (Duan *et al.,* 2011). In *V. vulnificus* biotype 1, the flagellum has been proved to be directly involved in

pathogenesis since diverse flagellin gene-mutants presented a significant reduction in motility, adhesion and cytotoxicity concomitantly to an attenuation of virulence and capacity of invasiveness *both in vivo* and *in vitro* (Kim and Rhee, 2003; Lee *et al.,* 2004a). No similar study has been performed in the zoonotic variant of *V. vulnificus.*

Adhesins are specialized surface proteins that mediate bacterial adhesion and are located on the bacterial surface or at the end of pili. Adhesins bind specifically to receptors on the surface of target host cells, determining tissue tropism of the pathogen and having a role in bacterial colonization of host cells (Klemm and Schembri, 1999). There are not many studies on adhesins in *V. vulnificus*. The pathogen possesses 2 types of type IVa pilus (MSHA and the chitin-regulated) and one of type IVb pilus (Flp) (Aagesen and Häse, 2012), but the only characterized has been the chitin-regulated. In 1998 Paranjpye and cols. (Paranjpye *et al.*, 1998) obtained a mutant in a clinical isolate of biotype 1 that was deficient in a peptidase/N-methyltransferase *pilD* (originally designated *vvpD*). This enzyme converts pre-pilin of a type IV pilum in mature pilin. The mutant did not form pili, was unable to secrete several extracellular degradative enzymes, and was significantly less adhesive to and cytotoxic for eukaryotic cells, as well as less virulent, although plasma resistance and capsule formation were not affected. The same research group characterized the phenotype of a second mutant deficient in pili, a *pilA* mutant deficient in the main pilin of the pilus, which showed reduced ability to form biofilms, lower adherence to HEp-2 epithelial cells and virulence for iron-overloaded mice (Paranjpye *et al.,* 2005). Both works confirm the important role that adhesion proteins have in pathogenicity of *V. vulnificus* biotype 1 to humans. No study on adhesins has been performed in the zoonotic variant of *V. vulnificus*.

2.   **Bacterial surface**

The capsule is a virulence factor for *V. vulnificus* biotype 1 whose putative function is to protect the microorganism from the bacteriolytic action of human plasma complement and from phagocytosis, facilitating the spread of the pathogen in blood and the invasion and colonization of internal organs (Simpson *et al.,* 1987;

Wright *et al.,* 1990). The encapsulated cells form opaque colonies on agar plates and the non-capsulated translucent ones (Simpson *et al.,* 1987). There have been described various serological types of capsules in *V. vulnificus* biotype 1, but all of them seem to act in the same way in the host, protecting from the innate immunity. The biotype 2 cells also produce capsules and opaque and translucent isoforms can spontaneously revert to the opposite in the laboratory in a similar rate to that of biotype 1 (Biosca *et al.,* 1993).

Eel virulence assays demonstrate that the capsule is not essential for eel virulence by the i.p. route because the translucent variants are still virulent (Biosca *et al.,* 1993). Nevertheless, the loss of capsule increases LD_{50} in 1 log unit, suggesting that the capsule exerts some role in the virulence for eels (Biosca *et al.,* 1993). Hence, the capsule is not required for resistance to the bactericidal effect of fresh eel plasma and phagocytosis by eel granulocytes, although the translucent variant grows less efficiently in fresh eel plasma, fixes more complement and is more sensitive to the antimicrobial peptides than the corresponding capsulated isoform, which could explain the 1 log attenuation in virulence (Valiente *et al.,* 2008d). In contrast, the capsule seems to be definitely required for virulence through water, the natural route for fish infection, since the translucent variants are completely avirulent by immersion challenge (Amaro *et al.,* 1995). Related to this, experiments performed *in vitro* and *in vivo* demonstrate that the translucent isoform colonizes the gills *in vivo* less efficiently than the opaque isoform, resulting in a population size that could be even below the minimum needed for a successful infection (Amaro *et al.,* 1995, Valiente *et al.,* 2008d) (Figure 9). Thus, the capsule appears to be a factor needed for successful gill colonization more than to eel invasion.
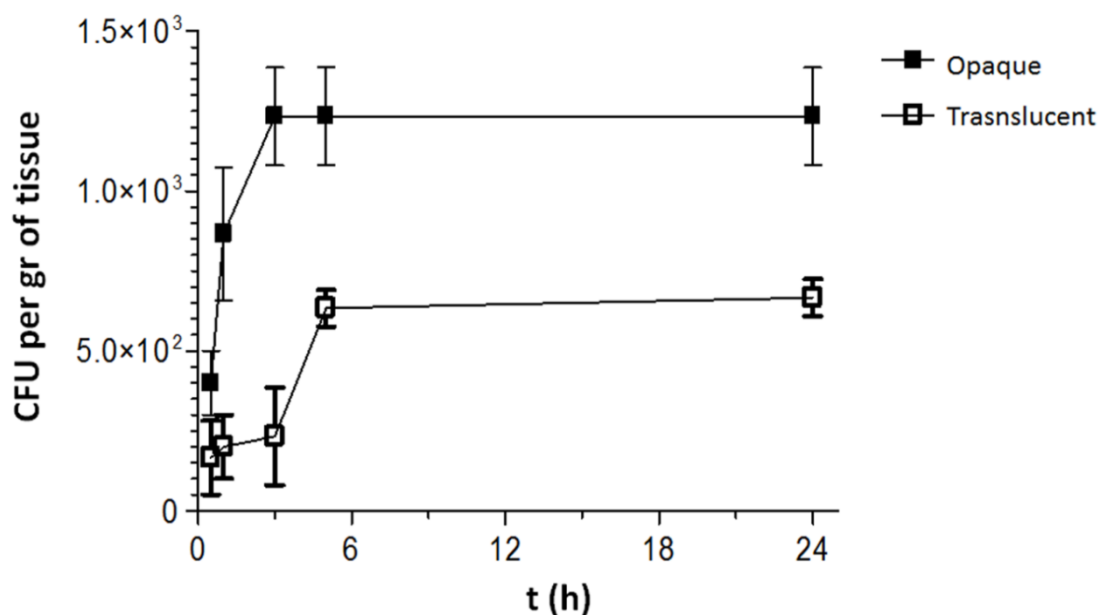
Figure 9. Capsule and *in vivo* gill colonization. Bacteria recovered from gills (CFU/gr) after immersion challenge with the two variants, opaque (with capsule) and translucent (without capsule), of the biotype 2-serovar E strain CECT4999. Image from Amaro *et al.* (1995).

The comparison between the opaque and translucent variants of the zoonotic serovar of biotype 2 for their virulence in mice by the i.p. route and resistance to human plasma demonstrates that the capsule is absolutely essential for these two properties (Amaro *et al.,* 1994). In consequence, the capsule of the zoonotic strain seems to act as an invasion factor in mammals, as has been described for biotype 1. These results suggest that some differences exist in the innate immunity displayed by mammals and teleosts and, as a result of this, different virulence gene sets are used by *V. vulnificus* to trigger septicaemia in mammals and teleosts.

Amaro and cols. (Amaro *et al.,* 1994) suggested that LPS from serovar E strains may be involved in eel virulence and plasma resistance after testing a spontaneous rugose mutant in a series of *in vivo* and *in vitro* assays. A *gne* mutant, deficient in UDP-N-acetylgalactosamine (UDP- GalNAc) 4-epimerase activity, an enzyme that catalyzes the conversion of UDP-GalNAc to UDP-N-acetylglucosamine (UDP-GlcNAc), lacked O-antigen, suggesting that the O-polysaccharide chain of serovar E may contain GlcNAc residues in each of its repeated units (Valiente *et al.,*

2008d). This was later confirmed by characterizing the purified LPS of a biotype 2-serovar E strain (Knirel *et al.,* 2009). The O-antigen deficient mutant showed reduced swarming and swimming on semisolid surfaces in parallel to impaired biofilm formation on eel mucus (Valiente *et al.,* 2008d). In addition, the mutant showed deep alterations in the outer membrane architecture concomitantly to a noticeable increase in sensitivity to cationic peptides and fresh plasma from eels and humans as well as to the phagocytosis by eel phagocytes (Valiente *et al.,* 2008d). As expected, the O-antigen deficient mutant was completely avirulent for eels both by i.p. and immersion routes, and was almost avirulent for mice by the i.p. route. These results confirm the multi-factorial role of the O-antigen of *V. vulnificus* biotype 2-serovar E in virulence for teleosts and mammals. Thus, the O-antigen acts as colonization factor in fish by facilitating the attachment and biofilm formation on the gills, and as an invasion factor in fish and mammals by protecting the bacteria from common factors present in plasma and from phagocytosis. Interestingly, the O-deficient mutant can grow efficiently in complex media and, therefore, could be a good candidate for the development of a live vaccine against eel vibriosis, at least against those caused by the zoonotic serovar of *V. vulnificus* biotype 2.

3.   Exoenzymes: metalloprotease VvpE.

The metalloprotease, VvpE, of *V. vulnificus* biotype 1 is an exoenzyme that has been extensively studied (Miyoshi *et al.,* 1987). This protease belongs to the thermolysin family (Miyoshi and Shinoda, 2000). VvpE exhibits a broad range of biological activities: i) it can degrade multiple host proteins, such as structural (laminin, elastin and collagen) and serum (transferrin) proteins, heme (hemoglobin) and intestinal mucus proteins (lactoferrin IgA) (Miyoshi *et al.,* 1999; Kim *et al.,* 2007; Nishina *et al.,* 1992; Okujo *et al.,* 1996b); ii) it can increase vascular permeability and cause serious hemorrhagic damage after being injected in mice (Miyoshi *et al.,* 2000); iii) it can inactivate the human hemolysin (Shao and Hor, 2000). However, the role of *vvpE* in human pathogenesis remains unclear, as VvpE-deficient mutants show comparable, or higher, virulence than wild-type strains in mouse models (Shao and Hor, 2000). Recent studies showed the *vvpE* gene is highly

conserved among biotypes, serovars or phylogroups, suggesting a common role of VvpE for all biotypes (Valiente *et al.,* 2008a).

Valiente *et al.* (Valiente *et al.,* 2008a) discovered that, in a biotype 2-serovar E strain, and contrary to what was expected, VvpE was involved in fish colonization, but not in invasion and lesion formation. This conclusion is supported mainly by the fact that the mutant strain was avirulent by immersion while it caused the classical warm-water vibriosis, with all the associated clinical signs (including the hemorrhages), and the same LD_{50} as the wild-type strain after i.p. injection. Eel colonization experiments highlighted the importance of the mucus that covers the gills in the colonization process since *V. vulnificus* biotype 2-serovar E was able to attach to the gills only when the cells or gills are covered with mucus (Figure 10). Additional experiments of chemotaxis and attachment to different types of fish mucus (skin, gill and gut mucus) showed that the wild-type strain was chemo-attracted and attached significantly better than the mutant strain to all types of mucus, especially the gill mucus (Figure 10) (Valiente *et al.,* 2008a).
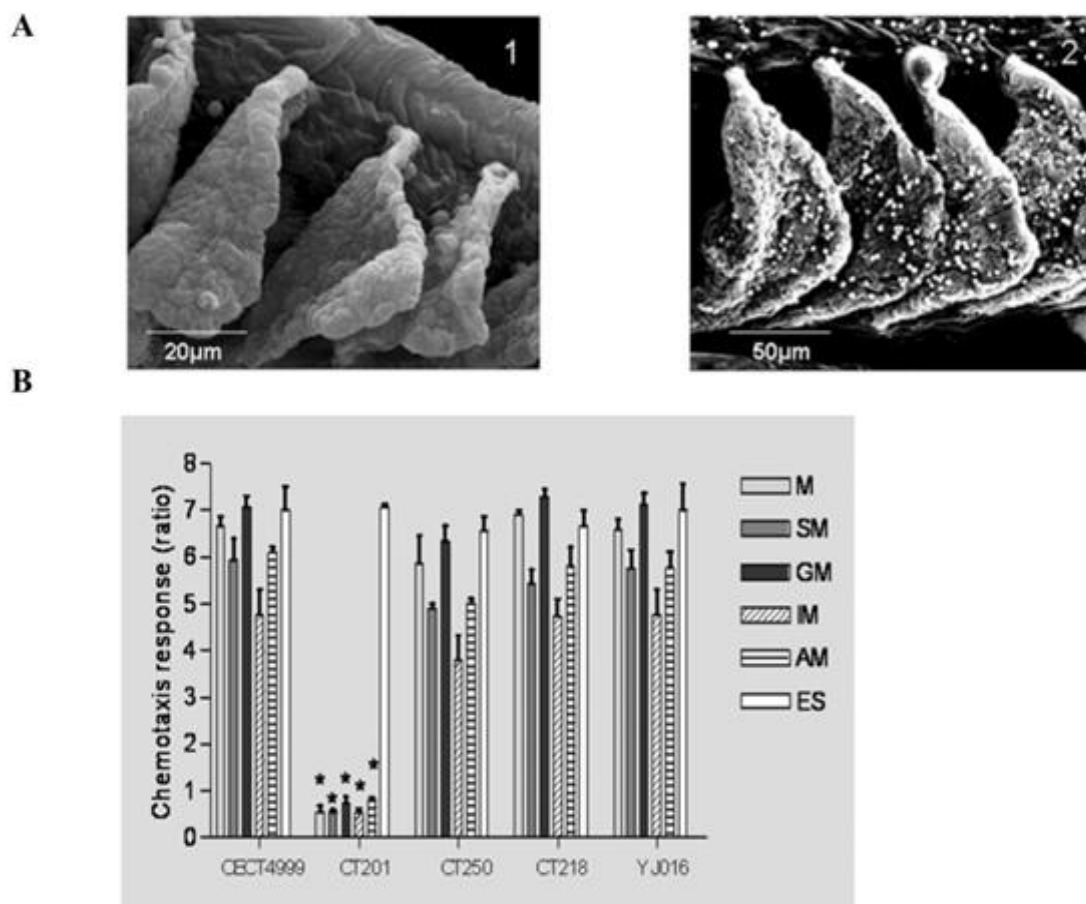
Figure 10. **Role of mucus and the protease Vvp in the attachment to gills**. A) Electron micrographs of cultured gills incubated with the biotype 2-serovar E strain CECT4999 without (1) or with (2) mucus. B) Chemotaxis towards mucin (M), algae mucus (AM), eel skin mucus (SM), eel gill mucus (GM), eel intestine mucus (IM) and eel plasma (ES). CECT4999, the wild type strain; CT201, Δ*vvpE* mutant; CT250, CT201 complemented in trans with *vvpE*; CT218, cured strain; YJ016, a biotype 1 strain isolated from human blood. Chemotaxis was measured as the chemotaxis response (CR) ratio (Valiente *et al.,* 2008a). Asterisk: significant differences with the wild-type strain with $p<0.05$. Image from Valiente *et al.* (2008a).

4. Iron acquisition systems

Nutritional immunity is the most ancient system of defense against pathogens common to all vertebrates (Weinberg, 2009). Basically, it consists in metabolic adjustments in order to make iron unavailable to microorganisms, i.e. by increasing synthesis of the iron-binding proteins such as lactoferrin in the mucous tissues and transferrin (Tf) in blood. Iron is an essential element for almost all living

bacteria, being the high iron concentration one of the factors that contributes to the rapid growth in both the environment and inside the host (Hor *et al.,* 2000). This trait is especially relevant in the cases of patients that present a high iron concentration in serum, whose are significantly more susceptible to suffer bacterial infections, where those caused by *V. vulnificus* have an outstanding role. In fact, the hematological disorders characterized by elevated iron levels in serum are a clear risk factor in case of human vibriosis caused by *V. vulnificus* (Strom and Paranjpye, 2000). To overcome the iron restricted conditions imposed by hosts, bacteria have evolved acquiring systems that allow them to sequester iron from host proteins and use it for their metabolic processes.

a)   Siderophores

One of the strategies is the based on siderophores, low-molecular weight compounds with a high binding affinity for iron that are produced by bacteria and fungi in iron starvation conditions. Siderophores can sequester iron in ferric form from iron-containing host proteins for bacterial growth (Winkelman, 2002). There are more than 500 types of bacterial siderophores classified into the following categories depending on the functional group that binds the ferric ion and the formed complex (Crosa and Walls, 2002):

1. Catechols
2. Hydroxamates
3. A-hydroxicarboxilic acids
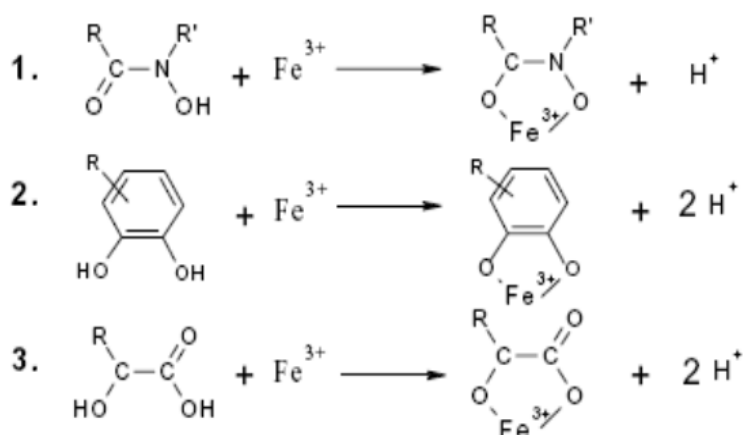
Siderophores show a value of ferric ion-binding constant between 10^{22} and 10^{55}, value that is higher than that of proteins like transferrin or ferritin, allowing siderophore to catch Fe^{+3} directly from these host proteins (Neilands *et al.,* 1995). This binding is specific because siderophores can discriminate iron from aluminum, calcium, copper and zinc. The mechanism by which the siderophores are internalized to the cytoplasm after scavenge iron from host proteins is detailed in Figure 11.

V. vulnificus biotype 1 produces a siderophore of catechol type called vulnibactin (Okujo *et al.,* 1996a). The mutants deficient in vulnibactin production show attenuation in virulence degree (measured as LD_{50}) for mice around 1 and 2 log units (Litwin *et al.,* 1996). Some authors have described that some strains of biotypes 1 and 2 can produce a hydroxamate siderophore in addition to vulnibactin although the biosynthesis genes have not been identified (Simpson and Oliver, 1983; Biosca *et al.,* 1996a). Finally, some biotype 1 strains produce a system that can bind and internalize exogenous hydroxamates produced by other bacteria and the genetic basis for such system has been identified and described (Tanabe *et al.,* 2005).
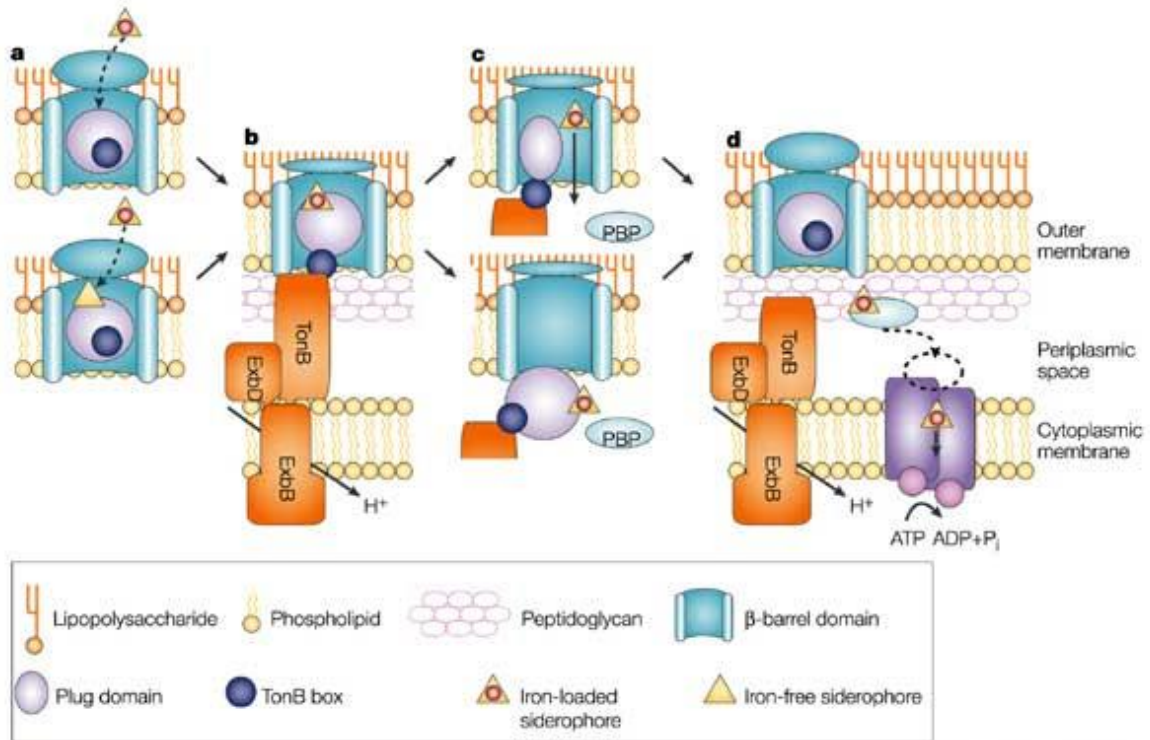
Figure 11. **Mechanism of internalization of ferri-siderophore complex to the cytoplasm**. A) The ferri-siderophore interacts specifically with the outer membrane receptor (it has been observed cases where the apo-form of the siderophore joins the receptor). B) The interaction triggers conformational changes in the structure of the receptor and leads to the interaction with TonB complex with the TonB box, a region of the receptor that contact directly with the TonB complex. C) The TonB complex induces conformational changes by using the protonmotive force in the outer membrane receptor thus releasing the ferri-siderophore to the periplasm (it is not clear if the ferri-siderophore passes across a channel or if it is translocated together with the plug domain). D) The periplasmic-binding proteins carry the ferri-siderophore to the ATP-binding casset (ABC) transporter of the inner membrane, that that finally internalizes it to the cytoplasm by an energy (ATP)-dependent process. Once in the cytoplasm, iron is released from the siderophore after a reduction process of Fe^{+3} to Fe^{+2} carried out by ferric reductases, since the affinity of the siderophore for Fe^{+2} is low. Image from Faraldo-Gomez y Sansom (2003).

b)   Heme/Hemoglobin receptors

Other host iron-containing proteins are the hemoproteins. The heme group is constituted by a protoporphirin IX molecule with a central iron atom (Figure 12). Heme is an important cofactor that is involved in many metabolic processes such as oxygen transport and energy production.
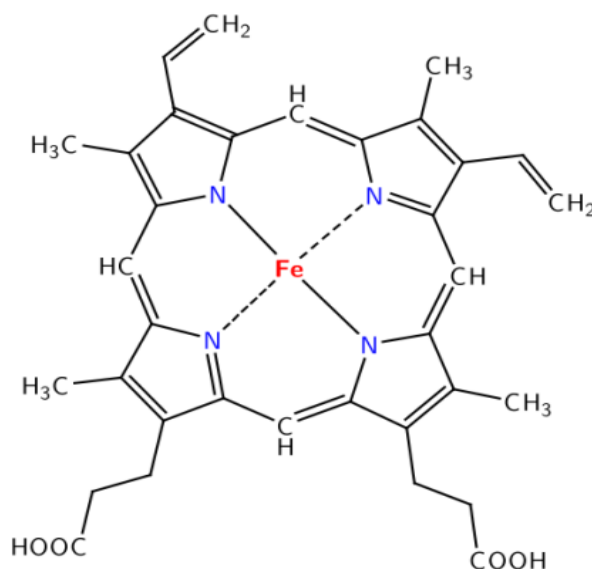
Figure 12. Structure of a protoporphirin IX molecule.

These classes of hemoproteins comprise hemoglobin, haptoglobin, hemopexin, cytochromes, catalases, peroxidases and albumin. Hemoglobin storage constitutes the major part of the cell iron content (up to 65%) and is considered an important source of iron for bacteria (Litwin and Calderwood, 1993).

To be able to use this iron, bacteria have developed outer membrane receptors that specifically recognize heme or hemoglobin, as well as low molecular weight compounds, hemophores, that are secreted and bound to the hemoglobin and carry it to the outer membrane receptor, to be posteriorly internalized to the cytoplasm and used as an iron source (Genco and Dixon, 2001). In Figure 13 is detailed the mechanism by which bacteria can acquire iron from hemoproteins.
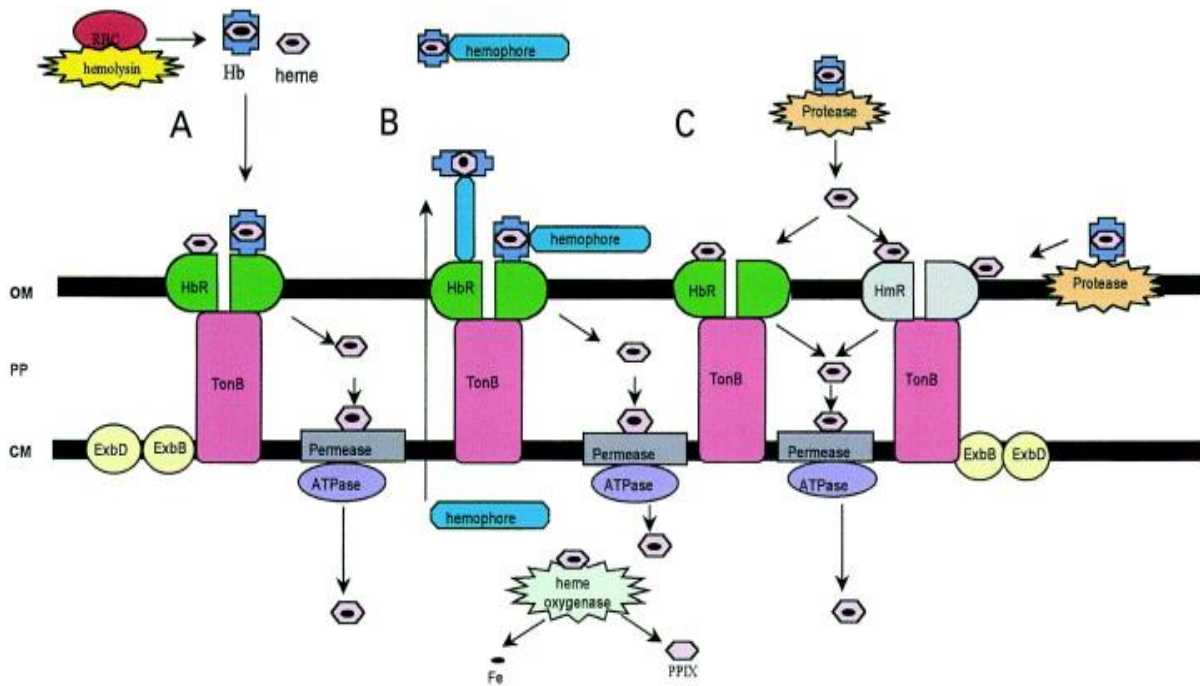
Figure 13. **Mechanism of utilizarion of heme/hemoglobin as iron source**. Hemin/hemoglobin can be released to the media by the activity of hemolysins against red blood cells and be internalizated by several ways. A) heme/hemoglobin interact with an outer membrane receptor that recognizes the ligand specifically. B) heme/hemoglobin binds to a hemophore that drives them to the outer membrane receptor. C) Extracellular or outer membrane proteases degrade the hemoglobin releasing heme group and leading to its binding to the outer membrane receptor. In all cases, the outer membrane receptor internalizes the heme to the periplasm and subsequently to the cytoplasm helped by the TonB complex and an ABC transporter in an energy-dependent manner, respectively. In the cytoplasm it is hypothesized that an oxigenase releases the iron from the protoporphirin IX. Image from Genko and Dixon (2001).

This system has been described in *V. vulnificus* biotype 1, where the expression of *hupA* gene was under control of Fur protein and a mutation in *hupA* decreased the virulence degree for mice (Litwin *et al.,* 1998; Oh *et al.,* 2009). Moreover, a new mechanism of heme uptake has been recently identified in *V. vulnificus*, but has only been characterized in the biotype 1 (Datta and Crosa, 2012).

c)   Transferrin receptors

Other iron acquisition system is the one dependent on transferrin outer membrane receptors. The best characterized transferrin receptor is that of *Neisseria*, being considered important iron acquisition system and virulence factor that determine the pathogenicity of bacteria (Renauld-Mongénie *et al.,* 2004). As explained in Figure 14, the receptor interacts specifically with transferrin and, since the receptor Fe^{3+} binding constant is higher than that of the transferrin, the iron is released from transferrin and internalized by using a similar mechanism to the explained for siderophores and heme receptors. In some bacteria, there can be a second outer membrane receptor protein that acts coordinately with the first one to scavenge iron from transferrin (Stokes *et al.,* 2005). This system has not been described in *V. vulnificus*
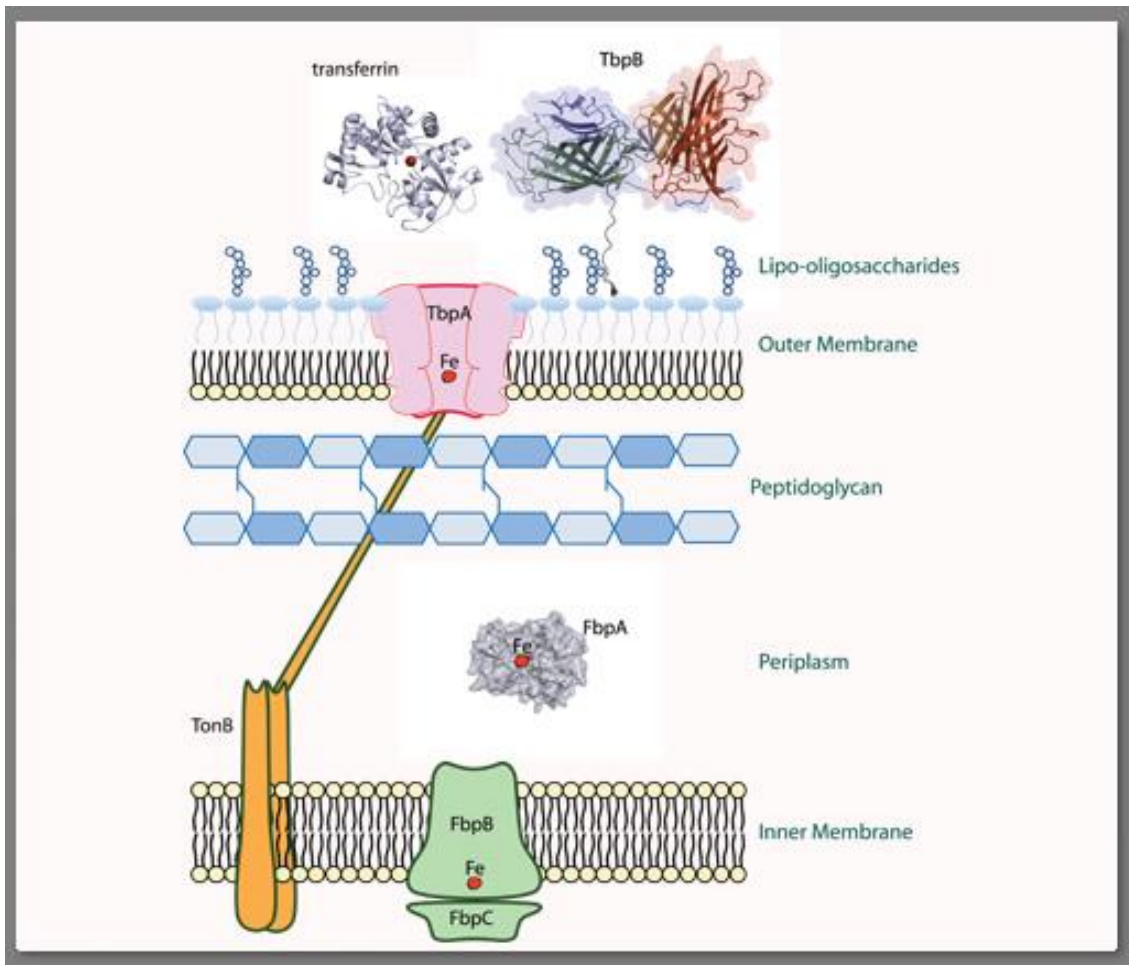
Figure 14. **Mechanism of utilizarion of holo-transferrin as iron source**. The transferrin binding protein A (TbpA) is an outer membrane receptor that interacts specifically with holo-transferrin. In some cases, a lipoprotein (TbpB) acts together with TbpA and contributes to the correct binding of transferrin directing the transferrin to the TbpA. The TonB complex provides the energy for this transport, and in the periplasm, iron binds to a Fbp (ferric binding protein) that drives it to the cytoplasm via a FpbB/C inner membrane transporter. Image from Trevor Moraes web page (http://biochemistry.utoronto.ca/moraes/).

5.   Lesional factors

a)   Hemolysin VvhA

The *vvhA* hemolysin of *V. vulnificus* is a cytolysin that has been reported to cause cytolysis of various eukaryotic cells including erythrocytes (Yamanaka *et al.,* 1990; Yamanaka *et al.,* 1987). The role of *vvhA* in pathogenicity of *V. vulnificus* is

controversial, basically because the *vvhA* deficient mutants obtained by different authors in biotype 1 strains are not attenuated in virulence degree for mice either by the intraperitoneal or intradermal route. However, these results contrast with the high toxic potency for mice of the purified VvhA; dose below 1 microgram per animal kill mice when are administered intraperitoneally (Lee *et al.,* 2004b). Due to this toxicity, many authors still work under the hypothesis that VvhA is one of the virulence factors that contribute to the pathophysiological lesions observed during *V. vulnificus* infection (Lee *et al.,* 2004b). Jeong and Satchell found that this cytotoxin collaborates with another one, the MARTX toxin (see later) to promote early *in vivo* growth in orally infected mice and dissemination of this pathogen from the small intestine to other organs (Jeong and Satchell, 2012). Using histopathological techniques, the authors find that both cytotoxins can cause villi disruption, epithelial necrosis, and inflammation in the mouse small intestine and that a double mutant, deficient in both cytotoxins is essentially avirulent, do not cause intestinal epithelial tissue damage, and is cleared from infected mice by 36 hours by an effective immune response (Jeong and Satchell, 2012).

b)   Multifunctional Autoprocessing Repeat in Toxin (MARTX)

The RTX toxin family is a group of cytotoxins produced by Gram-negative bacteria with a wide variety of functions. The RTX family is defined by two common features: characteristic repeats in the toxin protein sequences and extracellular secretion by the type I secretion system (T1SS). The name RTX (repeats in toxin) refers to the glycine and aspartate-rich repeats located at the C-terminus of the toxin proteins, which facilitate export by a dedicated T1SS encoded within the *rtx* operon. RTX have been related to virulence for mammals and/or resistance to amoebal predation (Satchell, 2011). There is a group of RTX, named MARTX (Multifunctional Autoprocessing RTX) that is present in at least eight gram-negative species including *V. vulnificus*. MARTX are modular toxins much larger than RTX toxins that are exported by modified type 1 secretion systems containing an additional ABC-transporter. MARTX contain external modules that are conserved at protein sequence level and an internal module subdivided into the functional domains that are the responsible of the toxic activity. The accepted model for MARTX action

(Satchell, 2007) consists in that the external modules interact with the membrane of the target eukaryotic cell, a pore is formed in the membrane and the functional domains of the internal module are translocated into the cytoplasm. Finally, a common CPD (cysteine protease) domain processes the toxin releasing active domains to the cytosol, which can move freely through the cell to access cellular targets (Figure 15). The repertoire of activities carried by these multifunctional toxins would ultimately be dictated by the selection of activity domains that they carry.
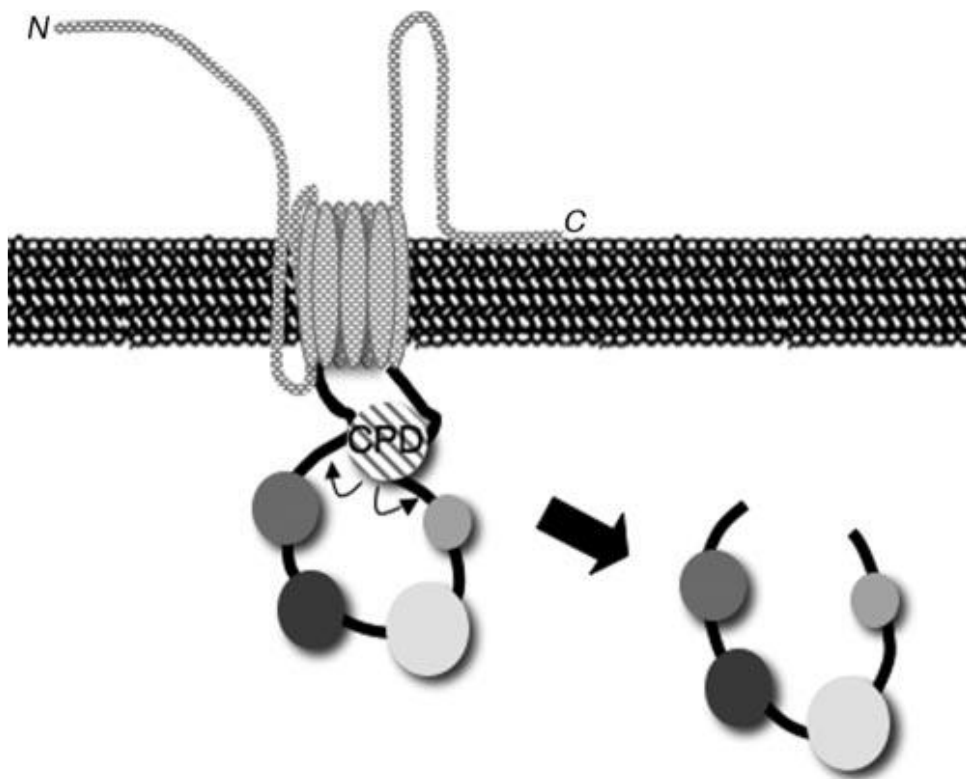
Figure 15. **Mechanism of action of MARTX toxins**. It is proposed that the N- and C-terminal repeat regions form a structure within the eukaryotic cytosolic membrane that is necessary to translocate centrally located activity domains across the membrane. Upon transfer of the CPD into the cytosol, the toxin would be autoprocessed, releasing active domains to the cytosol, where they could move freely through the cell to access cellular targets. Image from Satchell (2007).

MARTX of *V. vulnificus* is a widely recognized virulence factor involved in toxic action against intestinal epitheliocytes and phagocytic cells from the innate immune system (Lee *et al.,* 2007a; Kim *et al.,* 2008). As we have previously discussed, this toxin seems to act additively with VvhA and both are essential for mice virulence of *V. vulnificus* biotype 1 by the oral route (Jeong and Satchell, 2012). *V. vulnificus* species produces at least three different types of MARTX (types I-III) that differ in the functional domains of the internal module (Roig *et al.,* 2011; Kwak *et al.,* 2011). The three types present in the virulent strains are shown in Figure 16. The biotype 2 strains produce MARTX type III, regardless the serotype (Roig *et al.,* 2011). The gene encoding this toxin, *rtxA1₃,* is present in duplicate both in the chromosome II and in the virulence plasmid. MARTX type III mainly differs from types I and II in that possesses an actin-cross-linking domain or ACD, present in MARTX of *Vibrio cholerae* but absent in the rest of MARTX of *V. vulnificus* (Roig *et al.,* 2011).
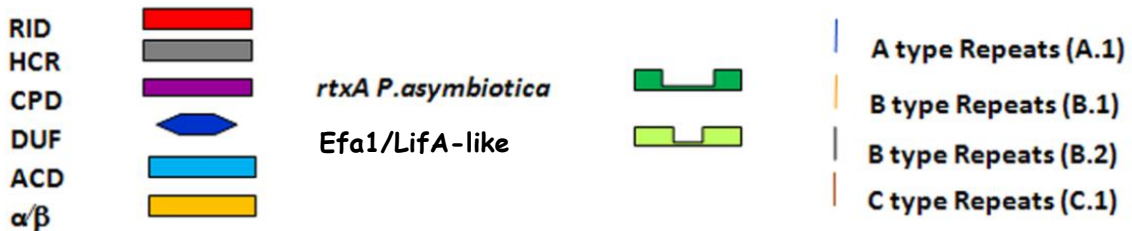
Figure 16. The protein structure of the MARTX types I (approx. 5200 aa), II (approx. 4700 aa) and III (approx. 4600 aa). The external regions, the repeats (vertical lines) and the internal domains for each toxin are color coded as indicated at the bottom. The putative domains are: RID, Rho-GTPase inactivation; HCR, highly conserved regions; CPD, autocatalytic cysteine protease; DUF, domain with an unknown function; ACD, actin cross-linking; α/β hydrolase; *rtx P. asymbiotica*, *rtxA* domain of *Photorhabdus asymbiotica*; Efa1/LifA, lymphostatin. Diagrams are drawn to scale. Figure adapted from Roig *et al.* (2011).

The phylogenetic analysis performed by Roig and cols. (Roig *et al.,* 2011) with the complete *rtxA1* gene sequences shows that *V. vulnificus* forms a well-supported, monophyletic group with two main subgroups, one corresponding to biotype 2 and the other to biotypes 1 and 3. This result suggests that, at least for this gene, biotype 2 has an evolutionary history different from that of biotypes 1 and 3, and that biotype 3 has emerged within the biotype 1 group.

According to the phylogenetic study of Sanjuán and cols. (Sanjuán *et al.,* 2011), biotype 2 is polyphyletic and has emerged by acquisition of a virulence plasmid by commensal isolates from fish-farms. Kwak and cols. (Kwak *et al.,* 2011) and Roig and cols. (Roig *et al.,* 2011) found evidences that support the hypothesis that the acquisition of the virulence plasmid would have favoured a recombination process between the chromosomal and plasmid *rtx* genes giving rise to a new variant of the mosaic gene that has probably been advantageous for the bacterium in the fish farming related environment. The presence of identical duplicated genes in the genome of the biotype 2 isolates suggests that either the acquisition has been recent, or a strong purifying selection is acting against mutations that modify the gene function.

IV. Virulence regulation

Coevolution between bacteria and their hosts determines characteristics of the interaction, the bacterial virulence genes involved, and the regulatory systems controlling expression of virulence genes. The association between *V. vulnificus* and their hosts appears to be a recent event since the first vibriosis cases were registered in the 70´s of 20th century. By this reason, the virulence-regulatory networks in *V. vulnificus* are in the first steps of an evolutive process probably from the commensal-regulatory networks involved in the relationships of this pathogen with their natural animal reservoirs. In this context, a series of virulence regulators have been studied in *V. vulnificus* biotype 1 taking as reference the model *V. cholerae*-humans.

A. Virulence transcription regulators

1. SmcR

Quorum sensing (QS) is a process of cell-cell communication that allows bacteria to share information about cell density and adjust gene expression

accordingly. This process enables bacteria to express energetically expensive processes as a collective only when the impact of those processes on the environment or on a host will be maximized (Xavier and Blassler, 2003). Among the many traits controlled by QS is the expression of virulence factors by pathogenic bacteria. The QS phenomenon has been poorly characterized in *V. vulnificus.* The biotype 1 harbors homologs of LuxPQ, a sensor for a borate diester autoinducer (AI-2), and carries a *luxS* gene, encoding the AI-2 synthase (Kim *et al.,* 2003b). A homoserine lactone autoinducer (AI-1) has been detected in some biotype 2 strains (Valiente *et al.,* 2009); however, in many other well-studied strains, such as YJ016, CMCP6, and MO6-24/O, whose genome sequences have been completely determined (Chen *et al.,* 2003; Kim *et al.,* 2003a; Park *et al.,* 2011), the effort to identify an AI-1 compound or a gene responsible for AI-1 biosynthesis has been unsuccessful. An analysis of the genome sequences of these three strains has uncovered homologs of *luxU* and *luxO*, which encode proteins responsible for the transduction of signals via a phosphorelay from a sensor protein (Roh *et al.,* 2006). These signals are funneled to the master regulator **SmcR**, a homolog of LuxR in *V. harveyi,* which subsequently induces the expression of *vvpE* (Jeong *et al.,* 2003)and represses *yegD*, a gene encoding a chaperone (Lee *et al.,* 2008b). SmcR also represses the expression of *hlyU* (Shao *et al.,* 2011) (see later), a gene encoding an activator that induces the expression of the virulence factors *vvhA* and *rtxA1₋₁* (Kwak *et al.,* 2011). Mutation of *smcR* gene alters multiple phenotypes in *V. vulnificus* biotype 1: biofilm formation, colony morphology, motility and survival under acidic or hyperosmotic stressing conditions as well as virulence to mice (Lee *et al.,* 2007b)

2.   HlyU

This protein belongs to a family of small metal-regulatory transcriptional repressors. The *V. vulnificus* HlyU of biotype 1 is a 11.9-kD protein that is very similar to the *V. cholerae* one (93% of similarity and 82% identity) (Liu and Crosa, 2012). The *in silico* modeled structure of *V. cholerae* and *V. vulnificus* HlyU shows that it do not have the key metal-sensing residues. It is thus possible that both HlyU evolved from an ancestral transcriptional repressor by loss of the metal-binding sites and they are the only member of this family that has a positive regulatory

function (Liu and Crosa, 2012). HlyU of *V. vulnificus* biotype 1 differs from that of *V. cholerae* in that it does not act as a direct transcriptional activator but instead HlyU acts as a derepressor of the global repressor H-NS, which in turn repressed the expression of *rtxA1-1* (and probably *vvhA*) (Liu and Crosa, 2012). Like other members of this family, *V. vulnificus* HlyU contains a helix-turn-helix motif and binds to DNA by forming a homodimer. According to the model of Liu and Crosa prior to the bacterium contacting the host cells, H-NS binds to multiple AT-rich upstream and downstream regions of the *rtxA1-1* operon promoter. The H-NS binding causes the DNA molecule to bend forming a DNA:H-NS:DNA bridge that either impedes the movement of RNA polymerase or excludes the entry of this enzyme thus repressing the expression of the *rtxA1-1* operon. Once the bacterium is ingested or invades open wounds, the bacterium contacts the host cells and somehow the expression of the HlyU protein is induced. HlyU binds to the upstream region of the *rtxA1-1* promoter and replaces some of the H-NS molecules interfering and breaking the DNA:H-NS:DNA structure, resulting in *rtxA1* gene expression (Liu and Crosa, 2012).

3.   cAMP receptor protein (CRP)

Bacteria have developed several mechanisms which allow the preferred utilization of the most efficiently metabolizable carbohydrates when these organisms are exposed to a mixture of carbon sources. Interestingly, the same or similar mechanisms are used by some pathogens to control various steps of their infection process. The efficient metabolism of a carbon source might serve as signal for proper fitness. Alternatively, the presence of a specific carbon source might indicate to bacterial cells that they thrive in infection-related organs, tissues or cells and that specific virulence genes should be turned on or switched off. The master regulator for this complex process is the cAMP receptor protein **(CRP)**. The system works as follows: the adenylate cyclase (Cya) catalyzes the conversion of ATP into cAMP, which in *Vibrio* forms a complex with the cAMP receptor protein CRP and allows it to bind to operator sites in front of numerous catabolic and virulence operons thereby stimulating (or in a few instances inhibiting) their expression. Thus, *V. vulnificus* biotype 1 CRP activates the expression of *vvhA* (Choi *et al.,* 2002), *vvpE* (Kim and Shin, 2010) and TonB3 (Alice and Crosa, 2012), a complex that

transduces the proton motive force of the cytoplasmic membrane to energize substrate transport across the outer membrane. In addition, a mutation in the *V. vulnificus crp* gene resulted in an impediment of bacterial growth and colony morphology was converted from opaque to translucent type, which implies a decrease in capsule production, besides showing significant decrease in motility and adhesion to host cells (Kim *et al.,* 2013a). The role of this global regulator is extended not only to virulence genes, but also to other global regulators involved in virulence, as Lee and cols. (Lee *et al.,* 2008c) demonstrated when detected that the cAMP-CRP complex bound to *rpoS* upstream region resulting in a repression of *rpoS* gene transcription. This regulator, **RpoS**, is a sigma factor involved in changes in bacterial physiology and structure, and has been shown to contribute for better survival under nutrient starvation, oxidative stress, UV irradiation and acidic conditions (Lee *et al.,* 2008c). RpoS has been shown to regulate several virulence factors in *V. vulnificus* as metalloprotease *vvpE* (Jeong *et al.,* 2001) as well as the transcriptional regulator *fur* (Lee *et al.,* 2003).

4. Fur

The Ferric Uptake Regulator (Fur) is a protein that controls the expression of most of the genes involved in iron transport and utilization, as well as other important cellular processes (Hantke, 2001). It is a ubiquitous protein in prokaryotes formed by 128-160 aminoacids divided in two monomers. The best characterized Fur protein is that of *E. coli* (Bagg and Neilands, 1987; Saito *et al.,* 1991). This protein has a molecular weight of 17 KDa and two distinct domains; the C-terminal domain, involved in iron binding and the interactions needed for dimerization, and the N-terminal domain involved in DNA binding. The classical mechanism by which Fur exerts its regulatory activity is explained in Figure 17; in presence of iron, Fur dimerizes acquiring the appropriate structure to interact directly with a DNA region, called furbox, in the promoter region avoiding gene transcription; in contrast, when iron concentration in media is low, Fur cannot dimerize avoiding the interaction with the furbox and leading to the gene transcription.
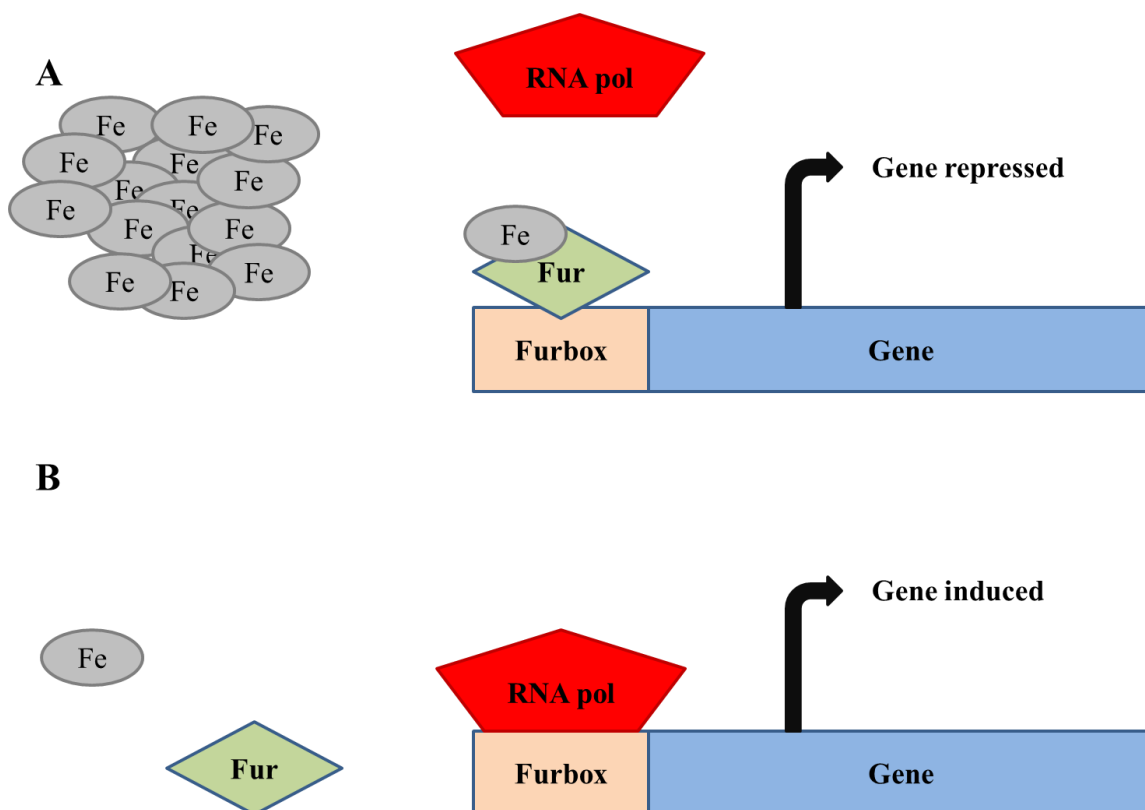
Figure 17. **Regulation mechanism of Fur protein.**

Although the classical process by which Fur controls gene expression is by repression in presence of exogenous iron, it has been shown that Fur may act as a repressor even in absence of iron (i.e. controlling iron storage molecule *pfr* and iron-dependent superoxide dismutase *sodB* in *Helicobacter pylori*, fumarate hydratase Cj1364 and hypothetical protein Cj0859 in *Campylobacter jejuni*) and even as activator (positive regulation) combined or not with iron (bacterioferritin gene *bfrB* and other genes in *Pseudomonas aeruginosa*) (all data recopiled from the review of Carpenter *et al.,* 2009) and controlling multiple genes in *Neisseria meningitidis* as microarray analysis suggested (Grifantini *et al.,* 2004).

The role of Fur as a global regulator, and not only a regulator of iron metabolism genes, is related with the important role that iron has in pathogenesis. Thus, many virulence-related genes are under Fur control, like those involved in toxin production, quorum sensing and biofilm formation in *P. aeruginosa, opa* genes

involved in adherence and invasion of hosts cells in *N. gonorrhoeae*, heat shock proteins in *N. meningitides*, hemolysin production in *V. cholerae*, the *irgA* gene, shiga toxins and hemolysin in *E. coli*, or vacuolating cytotoxin *vacA* in *H. pylori*. In fact, it has been demonstrated in a large number of bacteria that Fur mutation lead to a decrease in virulence degree. *H. pylori fur* mutant looses part of the colonization capacity on a murine model of infection due to an altered regulation of several genes involved in colonization. Other *fur* mutants in human pathogens as *Staphilococcus aureus* and *Listeria monocytogenes* showed reduced virulence in mammal models, and *Campylobacter jejuni* and *Edwarsiella tarda fur* mutants presented similar phenotypes in bird and fish animal models. Even in plant pathogen *P. syringae* the mutation of *fur* resulted in a decrease of virulence (all data recopiled from Carpenter *et al.,* 2009).

B.   Global virulence regulation in *V. vulnificus*

Virulence is a phenotype of a multifactorial nature, a complex feature of microbial pathogens that determines their survival and the interaction with their hosts. Virulence, in all its complexity, depends on the virulence factors, but as important is to possessing these factors as being able to coordinate them to develop the infectious diseases in the hosts in an optimal way.

To these end, the transcription regulators controls the expression of determined genes, but also there are described interactions between different transcription regulators that lead bacteria to respond in a much fine way to each situation to optimize energy and resources.

In Figure 18 are represented the main transcriptional regulators of *V. vulnificus* virulence genes and their interactions with the best characterized virulence factors, as well as with themselves.
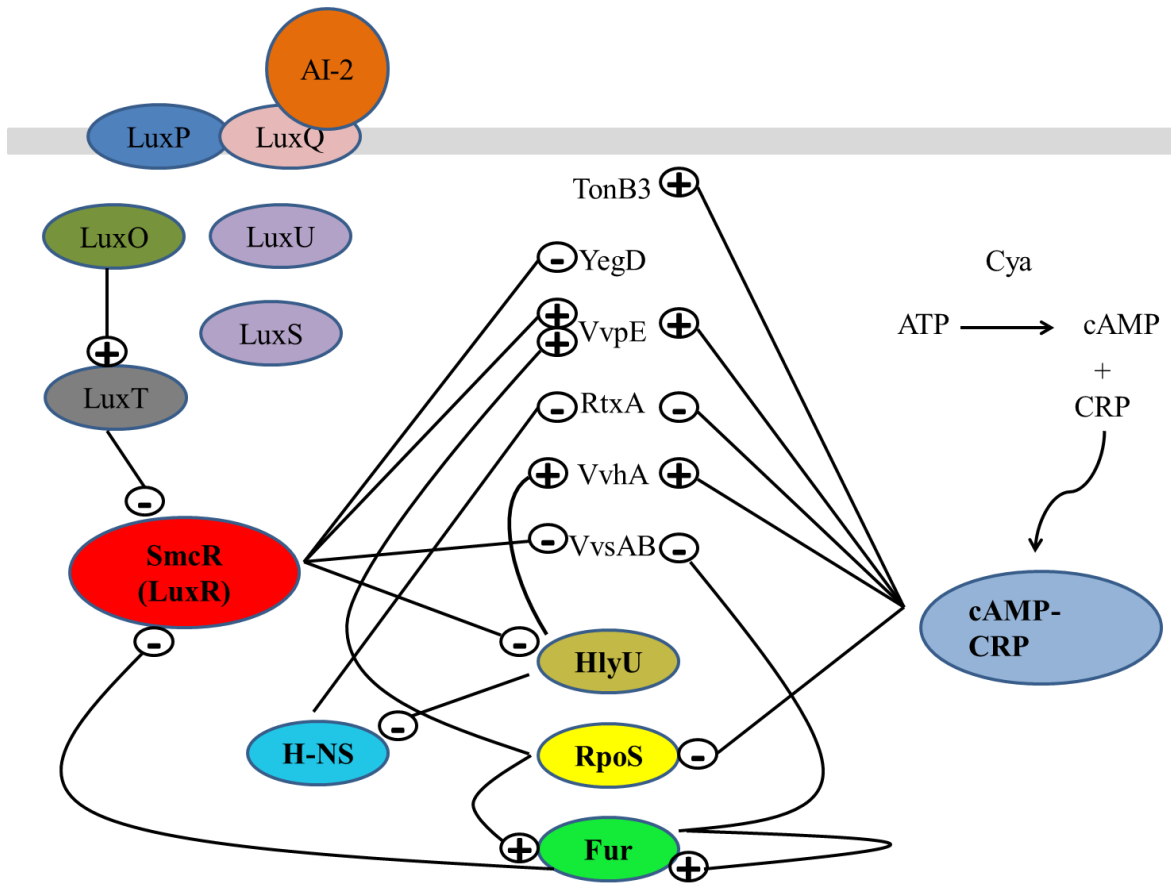
Figure 18. **Scheme of interactions between transcriptional regulators and virulence factors in *V. vulnificus*.**

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CHAPTER 1

MARTX OF THE ZOONOTIC

SEROVAR OF

Vibrio vulnificus

I. INTRODUCTION

The hallmark for *V. vulnificus* biotype 2 is the virulence plasmid pVvbt2. This plasmid contains only five genes that show significant homology to the previously described virulence genes (Lee *et al.,* 2008). These genes constitute an *rtx* gene cluster that contains two divergent operons; *rtxC-A1* encodes an RTX (repeats-in-toxin) (*rtxA1*) plus an enzyme for toxin modification, and *rtxB-D-E* encodes a toxin transport system (Lee *et al.,* 2008). RTX toxins have been related to virulence for mammals and/or resistance to amoebal predation in multiple bacterial species (Satchell, 2007; Satchell, 2011). The RTX of *V. vulnificus* biotype 2 belongs to the MARTX subfamily (multifunctional autoprocessing RTX) (Lee *et al.,* 2008). MARTX are proteins of high molecular weight that share a modular structure formed by two conserved external modules (N- and C- terminus) harboring the repeated motifs, and one variable internal module containing different functional domains related to the specific activity of the toxin (Satchell, 2011). The external regions interact with the membrane of eukaryotic cells allowing the translocation of effector domains to the cytoplasm. Upon translocation, one of the internal domains, CPD, cystein protease domain common to all MARTX, is activated to process the internal part of the protein and release the rest of the internal domains, which alter eukaryotic cell function (Satchell, 2007).

As explained in the introduction section, *V. vulnificus* produces at least three different types of MARTX (that will be called types I-III) (Kwak *et al.,* 2011; Roig *et al.,* 2011) (encoded by $rtxA1_1$, $rtxA1_2$ and $rtxA1_3$). The structure of MARTX types I, II and III, as well as the designation of the different domains, are shown in Figure 16 at the Introduction section. The pVvbt2-encoded MARTX corresponds to type III, which is structurally different to MARTX types I and II (Roig *et al.,* 2011). MARTX type III differs from types I and II in that it has an ACD domain and two copies of the Efa1/LifA domain (equivalent to McfDUF domain described by Satchell [2007]) flanking the common α/β domain. Interestingly, $rtxA1_3$ (that encodes MARTX type III) is present in all biotype 2 strains, regardless of their sub-phylogroup, in two

copies, one in pVvbt2 and the other in chromosome II (Lee *et al.,* 2008, Roig *et al.,* 2011).

The objective of the present chapter was to find out the reasons for the spreading of *rtxA1₃* among the biotype 2 strains and to characterize its role in this biotype. To this end we obtained single- and double-knockout mutants in the chromosomal and plasmid *rtxA1₃* genes, from a biotype 2-serovar E strain and used them in a series of *in vivo* and *in vitro* experiments with fish, freshly obtained fish cells and fish cell lines under the hypothesis that *rtxA1₃* is an essential gene for the survival of biotype 2 in the fish farming environment. We also included mice and human cell lines to test the potential role of this toxin in human virulence.

II. MATERIAL AND METHODS

A. Bacterial strains, growth media and conditions

The bacterial strains used in this study are listed in Table 1. The bacteria were routinely grown in LB-1/LBA-1 (Luria-Bertani broth/agar, 1% NaCl) or TSB-1/TSA-1 (tryptic soy broth/agar, 1% NaCl). Culture purity as well as the homogeneity of colony morphology were routinely tested on TSA-1 plates (Biosca *et al.,* 1993). In some experiments the bacteria were grown in CM9/CM9A (M9 minimal medium broth/agar supplemented with 0.2% casamino acids [Difco] and 0.3% yeast extract) (Sambrook and Russell, 2001), CM9-Fe (CM9 plus 100 μM FeCl₃), CM9-Hb (CM9 plus 10 μM bovine hemoglobin [Sigma]), CM9-Tf (CM9 plus 40 μM iron-free human apotransferrin [Sigma]) (Biosca *et al.,* 1996), CM9-HP (CM9 supplemented with human plasma [vol/vol], see later) and CM9-EP (CM9 supplemented with eel plasma [vol/vol], see later). In all cases, cultures were inoculated with an overnight starter culture in CM9 at a ratio of 1:100 (vol/vol) in a final volume of 5 ml and the growth curves were constructed from 0 to 24 h post-inoculation. *V. vulnificus* strains were incubated at 28ºC and *E. coli* strains at 37ºC for 18-24 h. The strains were stored in LB-1 plus glycerol (17%-20%) at -80ºC.

B.   Exotoxins and exoenzymes

The crude fraction of toxins and exoenzymes (Extra Cellular Products or ECPs) was obtained from 24 h-cultures on TSA-1 by the cellophane plate technique (Biosca and Amaro, 1996). Briefly, Trypticase soy agar plates supplemented with 1% NaCl were overlaid with sterile cellophane sheets and inoculated with 1 ml of an overnight culture of isolated colonies from each strain. After incubation for 24 h at 28ºC, cells were recovered with PBS (phosphate buffered saline, pH 7.0) and centrifuged at 13.000 rpm for 30 min at 4ºC. The resulting supernatants were sterilized by using nitrocellulose filters of 0.2 µm pore size (Millipore). Aliquots (1.5 ml) of each ECP sample were stored at -20ºC until used. ECP protein concentrations were determined by the Bradford Protein Assay (BioRad), using bovine serum albumin (BSA) (Sigma) as the standard.

C.   DNA/RNA manipulation.

1.   Genomic DNA purification.

DNA was extracted according to Ausubel *et al.* (2007) as follows:

- Centrifuge 1 ml of culture at 13000 rpm for 5 min at 4ºC
- Discard supernatant and resuspend pellet in 567 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8)
- Add 30 µl of SDS 10% and 3 µl of proteinase K (20 mg/ml) and mix by inverting tubes
- Incubate 1 h at 37ºC
- Add 100 µl of NaCl 5M
- Add 80 µl of CTAB (10%)/NaCl 0.7M and mix by inverting tubes
- Incubate 10 min at 65ºC
- Add 780 µl of chloroform/isoamyl alcohol (24:1, vol/vol), vortex vigorously and centrifuge 13000 rpm for 10 min at 4ºC
- Transfer upper phase to a new tube containing 1 volume of phenol/chloroform/isoamyl alcohol, vortex vigorously and centrifuge 13000 rpm for 10 min at 4ºC

- Transfer upper phase to a new tube containing 0.6 volumes of ice-cold isopropanol and incubate 30 min at -20ºC

- Centrifuge 13000 rpm for 10 min at 4ºC

- Wash pellet with ethanol 70%

- Dry and resuspend pellet in MiliQ water.

2. RNA extraction, DNAse treatment, cleaning and quantification

RNA was extracted from tissues (*in vivo* experiments) or mid-log phase cultures (*in vitro* experiments) with TRI reagent (Sigma) as follows:

- Centrifuge 1 ml of culture at 13000 rpm at 4ºC for 5 min

- Discard the supernatant and resuspend the pellet in 500 µl of Trizol (Sigma)

- Incubate with vigorous shaking at room temperature for 15 min

- Add 200 µl of chloroform

- Shaking briefly by vortex and incubate on ice for 5 min

- Centrifuge at 13000 rpm at 4ºC for 15 min

- Transfer upper phase to a new tube containing 300 µl of ice-cold isopropanol

- Shaking briefly by vortex

- Incubate on ice for 5 min and centrifuge at 13000 rpm at 4ºC for 15 min

- Discard the supernatant and wash the pellet with 1 ml of cold 70% ethanol

- Discard the ethanol, dry the tube and resuspend the pellet in 50 µl of DEPC water

RNA was subjected to a **DNase treatment** with the TURBO™ Dnase (Ambion) to digest and eliminate the residual DNA, following the manufacturer's instructions with a slight modification; the reaction time was prolonged to 45 min and DNAse treatment was carried out twice to ensure the digestion of all contaminant DNA.

To **clean the RNA** from salts, enzymes and residues of DNAse treatment, reactions were cleaned with the Rneasy® MinEute® Cleanup Kit (Qiagen) following the manufacturer's instructions.

RNA was **quantified** with a Nanodrop ND-2000 and only samples with A_{260}/A_{280} > 1.8 and A_{260}/A_{230} > 2 were selected.

3. Polymerase Chain Reaction (PCR) and quantitative-RT-PCR (qRT-PCR)

PCR was performed in 20 µl reaction volume that contained 0.2 µM forward and reverse primers, 1.5 U of Taq DNA polymerase (GoTaq; 5 U/µl; Promega), 4 µl of 5X Taq reaction buffer (Gotaq Green; Promega), 0.5 mM $MgCl_2$, 0.1 mM deoxynucleoside triphosphate (dNTP) mix (Promega) and 2 µl of DNA. The PCR was performed in a Techne thermocycler (TC-412). The reaction started with 10 min of denaturation at 94°C and was followed by 30 cycles of 40 s of denaturation at 94°C, 45 s of annealing at 50°C, and the extension time at 72°C depended on the amplicon length (generally, 1 min per kb). An additional extension at 72°C for 10 min completed the reaction. Amplicons were examined by agarose gel electrophoresis (1%) and ethidium bromide staining.

To quantify gene expression, cDNA was obtained from total RNA (1 µg per reaction mixture) with the M-MLV Reverse Transcriptase kit (Invitrogen) as described by the manufacturer. Quantification of cDNA was performed with Power SYBR® Green PCR Master Mix (Applied Biosystems) by using the StepOne Plus RT-PCR System (Applied Biosystems). Reactions were carried out in a final volume of 20 µl with 300 nM of forward and reverse primers, 2 µl of DEPC-water, 2 µl of cDNA and 10 µl of 2x Master Mix. The program used was 10 min of denaturalization at 95ºC followed by 40 cycles of 15 sec of denaturalization at 95ºC and 1 min of annealing and extension at 62ºC. Primers specific to *recA* (recA-F/recA-R: 5'CGCCAAAGGCAGAAATCG3' / 5'ACGAGCTTGAAGACCCATGTG3') and *rtxA1₃* (ACD-F/ACD-R: 5'GAGTGATGATGGGCGCTTTAC3' / 5'CAGCCGCGATGAGATG CT3') were used to amplify DNA fragments of about 60 bp. The threshold cycle (C_T) values were determined with StepOne Software V2.0 (Applied Biosystems) to establish the

relative RNA levels of the tested genes. DNA denaturing was conducted from 60 to 95ºC to obtain the melting curve for determining the PCR amplification specificity. For each tested gene, three independent bacterial cultures (or animals in the case of *in vivo* experiments) were subjected to RNA extraction and cDNA obtaining, and for each one, three measurements of cDNA were performed. The housekeeping gene *recA* was used as standard and the fold induction ($2^{-\Delta\Delta Ct}$) for each gene was calculated according to Livak *et al.* (2001).

All primers were designed from the genome of *V. vulnificus* YJ016 (chromosome 1 BA000037, chromosome 2 BA000038 and plasmid pYJ016 AP005352) and CECT4999 (virulence plasmid pR99 AM293858 and from unpublished sequences of chromosomes 1 and 2)

4.   Southern Blot

Southern hybridization was performed as described previously (Shao and Hor, 2000). Ten-microgram aliquots of the chromosomal DNA were completely digested with *Hin*dIII, fractionated by electrophoresis on a 1.2% agarose gel, and transferred to a nylon membrane (Hybond N+; Amersham Pharmacia Biotech). The probe was prepared and labeled with AlkPhos Direct Kit (GE Healthcare). The membrane was prehybridized for 30 min at 68°C, hybridized for 1.5 h at the same temperature, washed, and visualized by a chemiluminiscence scanner.

5.   Isolation of *rtxA1₃* mutant and complemented strains

The Δ*rtxA1₃* mutants were isolated by *in vivo* allelic exchange as previously described (Shao and Hor, 2000). Briefly, a DNA fragment amplified from CECT4999 with primers RTX7 (5'- CGGTAACGGCACAACCTTAG-3') and RTX10 (5'- CGCTTTCGCATCCACCAC-3'), was cloned into pGEMT®-easy vector (Promega). The region between two *Hin*dIII sites in this amplified DNA fragment was then removed by enzyme digestion and ligation to achieve excision of part of the Actin Cross-linking domain (ACD) and introduction of an early stop codon (Figures 1A). This recombinant DNA fragment was then cloned into pCVD442, a suicide vector,

between the *Sph*I and *Sac*I sites. This recombinant suicide plasmid was used to isolate the Δ*rtxA1₃* mutants by allelic exchange (Donnenberg and Kaper, 1991). The isolated mutants were checked by southern hybridization for their *rtxA1₃* genotype, either wild-type or with the deletion in the chromosome and plasmid (Figure 1B). The single mutants, Δp*rtxA1₃* (deletion in the plasmid, strain CT284) and Δc*rtxA1₃* (deletion in the chromosome, strain CT281), and the double mutant, Δp*rtxA1₃*Δc*rtxA1₃* (strain CT285), were thus identified. To restore the wild-type allele, an alternative strategy to complementation was used. This consisted of replacing the deleted allele in mutant CT285 with the wild-type allele through another allelic exchange to generate the complemented strain. In this case, a DNA fragment containing the sequence that was deleted in the mutants and its flanking regions amplified from strain CECT4999 by PCR with primers RTX13 (5'-GCGAGCTCGGTAACGGCACAACCTTAG-3') and RTX18 (5'-GCGAGCTCATCT CTGAGTGGAAG-3') was used instead. The growth of all the mutant and complemented strains in LB-1 was comparable to that of the wild-type strain (data not shown).
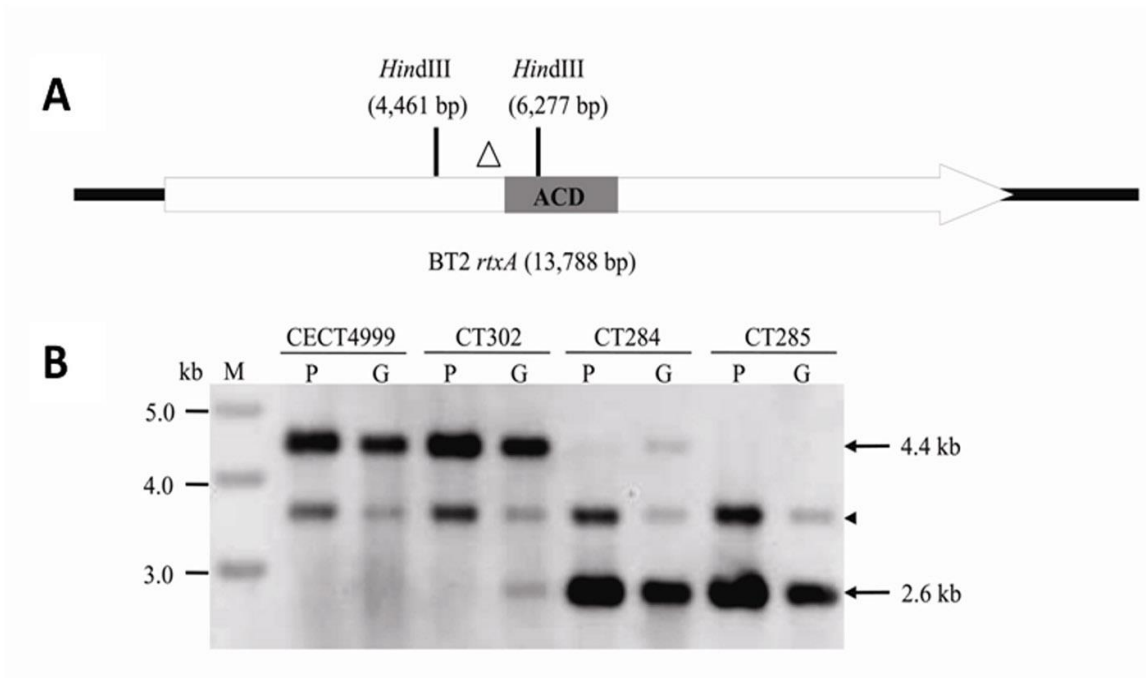
Figure 1. Confirmation of various *rtxA1₃* mutants. A) The gene structure of *rtxA1₃*. The coding region is indicated by an arrow. A 1,816-bp DNA fragment between the two *Hin*dIII sites that contains part of the putative ACD domain (5,886-7,269 bp) was deleted to generate the *rtxA1₃* mutants. The probe used in southern hybridization is indicated below. B) Southern hybridization analysis of the mutants. The plasmid DNA (P) or total DNA (G) was digested with *Bgl*II, separated in a 0.8% agarose gel, and probed with a DNA fragment amplified from *rtxA1₃* with primers RTX5 (5'-GAAACACGCAAAGCCGATGC-3') and RTX16 (5'-CTCATCTCTGAG TGGAAGCC-3'). CECT4999: wild-type; CT302: Δc*rtxA1₃*; CT284: Δp*rtxA1₃*; CT285: Δc*rtxA1₃*Δp*rtxA1₃*. The bands derived from *rtxA1₃* with and without deletions (2.6 kb and 4.4 kb, respectively) are indicated. M: 1 kb plus DNA markers. Image from Lee *et al.* (2012).

D. Phenotypic characterization of *rtxA1₃* mutant: *in vitro* assays

1. Cell damage assays with and without cell contact

Host cells. For these assays we used fish and mammal cell lines (**EP-1** [eel mucus-producing epithelial cells] [Kou *et al.,* 1995], **EPC** [Epithelioma papulosum cyprinid: this cell line was originally deposited as derived from carp, *Cyprinus carpio,* but finally identified by the ATCC –American Type Culture Collection- as derived

from fathead minnow, *Pimephales promelas,* ATCC CRL-2872] and **HEp-2** [human laryngeal carcinoma, ATCC CCL-23]) as well as freshly obtained human and eel erythrocytes. The fish cell lines were cultured in L-15 medium (Sigma) without CO_2 at 28ºC while the human cell line was cultured in DMEM medium (Gibco) with 5% CO_2 at 37ºC. Fresh eel and human erythrocytes, collected from blood by centrifugation (3000 rpm, 15 min, 4ºC) and washed three times with PBS were resuspended in L-15 at a proportion of 1% (vol/vol).

Citotoxicity. The cytotoxicity assays were performed with bacteria or their ECPs in 96-well plates containing $1 \times 10^{4-5}$ cells/well. The monolayers were infected with L-15-washed bacteria (harvested from a 4-h culture in L-15) at a moi (multiplicity of infection) of 10 or with ECP samples (vol/vol, 100 μl). The supernatant was collected from each well at 90 min post-infection and the cytotoxicity for cell lines was estimated by measuring the absorbance at 490 nm of released lactate dehydrogenase (LDH) while that for erythrocytes (see later) by measuring the absorbance at 540 nm of the released hemoglobin (Shinoda *et al.*, 1985). LDH assay was performed with the CytoTox 96 Non-Radioactive Cytotoxicity assay kit (Promega) following manufacturer's instructions.

Contact assay. To test whether contact with eukaryotic cells is essential for the expression of *rtxA1₃*, we used Transwell® culture plates with and without polycarbonate filters of 0.2 μm of pore diameter in the wells (Kim *et al.*, 2008). The lower chambers of the wells with filter were filled with 100 μl of a suspension of 10^6 eel erythrocytes in L-15 and the upper chambers with a suspension of 10^8 log phase cells from CECT4999 from a 4-h culture in L-15 (moi=100). In parallel, the wells without filter were filled with eel erythrocytes and bacterial suspensions in L-15 in the same proportions. The plates were incubated at 28°C for 3 h and samples were taken for quantification of *rtxA1₃* expression at 3 h post-infection.

2.   Amoeba predation assay

To obtain amoebae, the gills of moribund farmed fish (*Psetta maxima*) were washed several times with sterile filtered seawater (0.2 μm pore size) containing 80 mg/L of gentamicin. The branchial lamellae were then disaggregated by rubbing

them through a steel mesh, previously sterilized in an autoclave, into sterile seawater. After homogenization using a Pasteur pipette, a few drops of the mixture were placed onto agar plates (0.02 g of Difco Bactoagar ml–1 distilled water containing 20 mg of NaCl and heat-killed *E. coli*). The plates were then incubated at 21°C for 10 d, and examined daily with an inverted microscope to detect amoebae. Amoebae were cloned by removing a small square of agar containing trophozoites and transferring it to a fresh agar plate.

After amoebae culture, 3×10^4 viable trophozoites in 1 ml of marine amoeba medium (0.01% malt and yeast extract, 1% Difco Bactoagar in sterile filtered sea water) in the well of a microplate were coincubated with PBS-washed bacteria from an overnight culture in LB-1 at a moi of 1000. The viable amoebae were counted microscopically at different time intervals.

3.   Interference with the innate immune system

Obtention of plasma and erythrocytes and growth in plasma. Fresh eel plasma was obtained as described by Esteve-Gassent and cols. (Esteve-Gassent *et al.* 2004) and human plasma was purchased from Sigma. Humans were bled by vein puncture and eels were bled by cardiac or caudal fin puncture with a heparin-treated (50 mg/ml in a NaCl 0.9% solution) syringe, blood was centrifuged at 3000 rpm at 4ºC for 5 min and plasma and erythrocytes were separated in the supernatant and pellet, respectively. Bacterial resistance to plasma was assessed by mixing 100 µl of a bacterial suspension in PBS containing 10^3 CFU/ml with 100 µl of fresh plasma and the mixtures were incubated at 28ºC (for EP) or 37ºC (for HP) for 4 h with shaking (160 rpm). Samples were taken at 0 and 4 h post-incubation and the viable bacteria were enumerated by drop plate method (Hoben and Somasegaran, 1982).

Phagocytosis. Phagocytosis assays were performed with phagocyte-containing peritoneal exudate cells (PECs) freshly obtained from eels and a cell line of murine macrophages, RAW264.7 (ATCC TIB71). PECs were obtained from eels as described by Miyazaki and Kurata (1987). Briefly, eels of 300 gr of weight were injected with 5 ml of sterile protease peptone 10% (w/vol) in the peritoneal cavity

and maintained for 24 h in common storage conditions. The peritoneal cavity was washed with 5 ml of sterile cold saline solution (0.9% NaCl) and the phagocyte-containing peritoneal exudate cells (PECs) were collected by centrifugation at 1800 rpm at 4ºC for 5 min. PECs were resuspended in L15 supplemented with foetal bovine serum (FBS) (Sigma Aldrich) 10% (vol/vol) and with 100 µg/ml of primocin (Sigma Aldrich). Finally, PECs were seeded in a poli-L-lisyne treated 96-well plate (NUNC) at a concentration of 10^5 cells per well. After 18-24 hours, PECs were ready for infection. In parallel, RAW264.7 cells were cultured in poli-L-lisyne treated 96-well plates (NUNC) containing DMEM (Gibco) at 37ºC 5% CO_2 up to obtain a concentration of 10^5 cells per well.

The phagocytosis assay was performed as follows. Monolayers of RAW264.7 and eel PECs were inoculated with PBS-washed bacteria from a 4-h culture in L-15 at a moi of 10. After 0, 30, 60 and 90 min of co-incubation, two types of bacterial counts were performed: i) total bacteria that survived to the phagocytosis (externally and internally) and ii) the bacteria that were phagocyted. In the first case, the phagocytes were lysed with 0.1% Triton-X100 (RAW264.7) or 100µg/ml ice cold-saponin (PECs) with a 5 min incubation, and the bacterial number was determined by drop plate method. In the second case, the cells were treated with gentamicin (100 µg/ml, Invitrogen) for 30 min, washed with SS-1 and lysed with 0.1% Triton-X100/saponin for 5 min, and the released intracellular bacteria were enumerated by drop plate method. Finally, the intracellular survival rate after 90 min of interaction bacteria/phagocytes was determined by incubating with gentamicin, lysing the phagocytes after 30, 60 and 90 min of additional incubation and performing the corresponding bacterial counts.

For visualizing bacteria and phagocytic cells a hemacolor staining was performed as follows. Each well of a 24-well plate was covered with a poli-L-lysine-treated round coverslide and the plate was used to culture PECs as described before. The monolayer was infected with bacteria at a moi of 10. After 60 min of incubation, supernatant was discarded and coverslides were stained with hemocolor (Merc) as manufacturer's instructions. Observations and photos were made in a Nickon optic microscope.

E. Phenotypic characterization of *rtxA1₃* mutant: *in vivo* assays

1. Animal maintenance

Three populations of farmed European eel (*Anguilla anguilla*) of 10g, 20g and 100 g were used for virulence assays, colonization assays and blood extraction, respectively. The eels were purchased from a local eel-farm that does not vaccinate against *V. vulnificus*. Fishes were placed in quarantine in 170 L-tanks (6 fish of 100 g, 12 of 20 g or 20 of 10 g per tank, respectively) containing brackish-water (1.5% NaCl, pH 7.6) with aeration, filtration and feeding systems at 25 ºC for a week. After quarantine, healthy fish were distributed in 100 L tanks at the same ratio, were infected with *V. vulnificus* (see later) and were maintained for 1 week at the same maintenance conditions but without feeding. 6- to 8-week old BALB/c mice were purchased in Harlan Laboratory Models S.L. and maintained for 48 h in plastic cages of 100 L with water and feed provided by the Animal facilities of the University of Valencia (UV).

2. Virulence/Toxicity assay

The bacterial virulence and the toxicity of the ECPs for the eel, expressed as the LD_{50} (lethal dose to 50% of animal) or TD_{50} (toxic lethal dose to 50% of animal) value, was determined in European elvers of 8-10 g (Amaro *et al.,* 1995; Amaro *et al.*, 1994). The bacterial virulence for the mouse was determined in 6- to 8-week old BALB/c mice. The eels (by peritoneal injection or immersion) and the mice (by peritoneal injection) were infected with ten-fold serially diluted bacterial suspension or with different ECP dilutions. For both eels and mice, a total of six animals were used per control, strain and dose. In virulence assays, mortalities were recorded for 1 week only when the inoculated bacterium was re-isolated in pure-culture from the moribund animal. The LD_{50} was calculated as described (Reed and Münch, 1938) and expressed as CFU/g (i.p. injection) or ml of infective bath (immersion challenge), and the TD_{50} as µg of ECP/g.

3.   Colonization and invasion, and co-infection assay in eels

The eels were bath infected with the wild-type strain or with the double mutant strain at a dose equivalent to the LD_{50} of the wild-type strain. In the co-infection experiment, the eels were either injected with or immersed in a bacterial suspension containing equal numbers of the wild-type and the double mutant strains at a dose equivalent to the LD_{50} of the wild-type strain in each infection model. A total of 24 eels per strain were infected and 6 were immersed in the same conditions in PBS-1. 12 live eels were randomly sampled at 0, 9, 24 and 72 h, at a ratio of 3 animals per sampling point (Valiente and Amaro, 2006). The bacterial number per ml (blood) or g (gills, liver, kidney and spleen) of sample was estimated by the drop plate method. The bacteria recovered from the internal organs were checked by colony hybridization (Roig and Amaro, 2009) with two DNA probes, one for *vvhA* and the other for *rtxA1₃*, to determine their identity. The probe for *vvhA* was amplified with vvhA-F (5'CGCCACCCACTTTCGGGCC3') and vvhA-R (5'CCGCGGTACAG GTTGGCGC3'); that for *rtxA1₃* was amplified with rtxA1₃p-F (5'GCTCGATGGCGTTCAACG3') and rtxA1₃p-R (5'GCATCACGATCACCACGCGA 3').

4.   Histopathology of eel tissues

The tissues of bath infected eels with the wild-type strain or with the double mutant strains were examined by transmission electron microscopy (TEM); samples were fixed in cold 1% formaldehyde plus 2% glutaraldehyde in phosphate buffer 0.1 M, pH 7.4, for at least 6 h, and postfixed in 2% OsO_4 in the same buffer. After dehydration through a series of alcohol solutions (50%-100%), the tissues were embedded in araldite (Durcupan-Fluka). Semithin sections of 1 µm thick were stained with toloudine blue and observed under a light microscope to select the area of interest. Ultrathin sections of 0.120 µm thick obtained with an ultramicrotome (Leica) were stained with uranyl acetate and lead citrate, and examined by TEM (Jeol-1010).

F.   Statistical analysis

All the experiments were performed by triplicate and the significance of the differences was tested by using the unpaired Student's t-test with a $P < 0.05$.

III.   RESULTS

A.   Virulence and toxicity

The virulence of the wild-type strain and its derivatives was assayed in the eel and mouse. As shown in Table 1, the single mutants showed the same virulence degree for eels as the wild-type strain while the double mutant was completely avirulent by either i.p. injection or immersion. When the strains were tested in mice, the LD_{50} of the double mutant was about two-log units higher than that of the wild-type strain, while the LD_{50} of the single mutants was 3- to 8-fold higher than that of the wild-type strain (Table 1). As expected, the plasmid-cured strain (CT218) was avirulent for eels (the loss of the plasmid makes the bacterium sensitive to the eel innate immunity [Valiente *et al*., 2008]) and as virulent for mice as the wild-type strain while the plasmid-cured strain with Δ*crtxA1₃* (CT281) showed the same changes in virulence degree as the double mutant (Table 1). In addition, the single complemented CT310 (Δp*rtxA1₃* complemented) and the double complemented CT316 (Δp*rtxA1₃* and Δ*crtxA1₃* complemented) exhibited the wild-type level of virulence in both eel and mouse (Table 1). This result confirmed that attenuated virulence was not caused by an unexpected mutation that had occurred elsewhere.

The ECPs from the wild-type, the cured and the double mutant strains were equally toxic for eels, exhibiting similar mean toxic dose (TD_{50}) values (Table 1). This result suggests that MARTX type III, if present, is not active in the ECPs and that other *Vibrio* toxins could contribute to eel virulence.

Table 1. *V. vulnificus* biotype 2 strains used in this study; virulence, toxicity and resistance to the bactericidal effect of fresh eel plasma (EP) or fresh human plasma (HP).

Strain	Description	Reference	Virulence LD_{50} [a]			Toxicity TD_{50} [b]	Plasma Resist. [c]	
			mice (i.p.)	eel (i.p.)	eel (imm.)	eel (i.p.)	EP	HP
CECT4999	Wild-type strain	Lee *et al.,* 2008	5.7×10^5	1.5×10^2	1.5×10^6	1.8	++	+
CT218	Plasmid-cured CECT4999	Lee *et al.,* 2008	6×10^5	$>1 \times 10^7$	$>1.0 \times 10^8$	1.7	-	+
CT281	CT218 Δ*crtxA1₃*	This study	ND	$>1 \times 10^7$	$>1.0 \times 10^8$	ND[d]	ND	ND
CT284	CECT4999Δp*rtxA1₃*	This study	4.7×10^6	1.5×10^2	1.5×10^6	ND	++	+
CT285	CECT4999Δp*rtxA1₃*Δ*crtxA1₃*	This study	5.4×10^7	$>1.7 \times 10^7$	$>7.0 \times 10^7$	1.8	++	+
CT302	CECT4999Δ*crtxA1₃*	This study	1.7×10^6	1.7×10^2	2×10^6	ND	++	+
CT310	Complemented from CT285 (Δp*rtxA1₃* complemented)	This study	ND	3.5×10^2	ND	ND	++	+
CT316	Complemented from CT285 (Δp*rtxA1₃* and Δ*crtxA1₃)*	This study	5×10^5	3×10^2	ND	ND	++	+

[a] Virulence was calculated as LD_{50} after intraperitoneal injection (i.p.) (CFU per animal) or bath immersion (imm.)(CFU per ml).

[b] Toxicity degree is expressed as TD_{50} (µg of ECP per g fish).

[c] Bacterial growth after 4 h of incubation in fresh eel plasma (EP) and human plasma (HP) is expressed as the ratio final *vs* initial counts (-, no growth; +, between 1 and 10; ++, between 10 and 100; +++, between 100 and 1000).

[d] ND, not done.

B.   Eel colonization and invasion

To examine whether the *rtxA1₃* gene plays a role in fish colonization and invasion, eels were infected by immersion with the wild-type and the double-mutant strains. Contrary to expected, the double-mutant was not visibly deficient in eel colonization and invasion capacity (Figure 2A). Thus, it was able to adhere to gills, establishing a Table population similar in size to that of the wild-type strain, and cause septicemia but without killing the eels (Figure 2A). In addition, bacterial population size in the internal organs did not differ significantly to that of the wild-type strain at 9, 24 and 72 h post-infection (Figure 2A). This result strongly suggests that MARTX type III is a lethal factor for eels. Additional co-infection experiments with both the wild-type and double-mutant strains by immersion revealed that the former, except from gills, was recovered in higher proportions from the blood and head-kidney (Figure 2B), which suggests that MARTX type III could also confer some advantages to the bacterium during eel colonization and invasion.
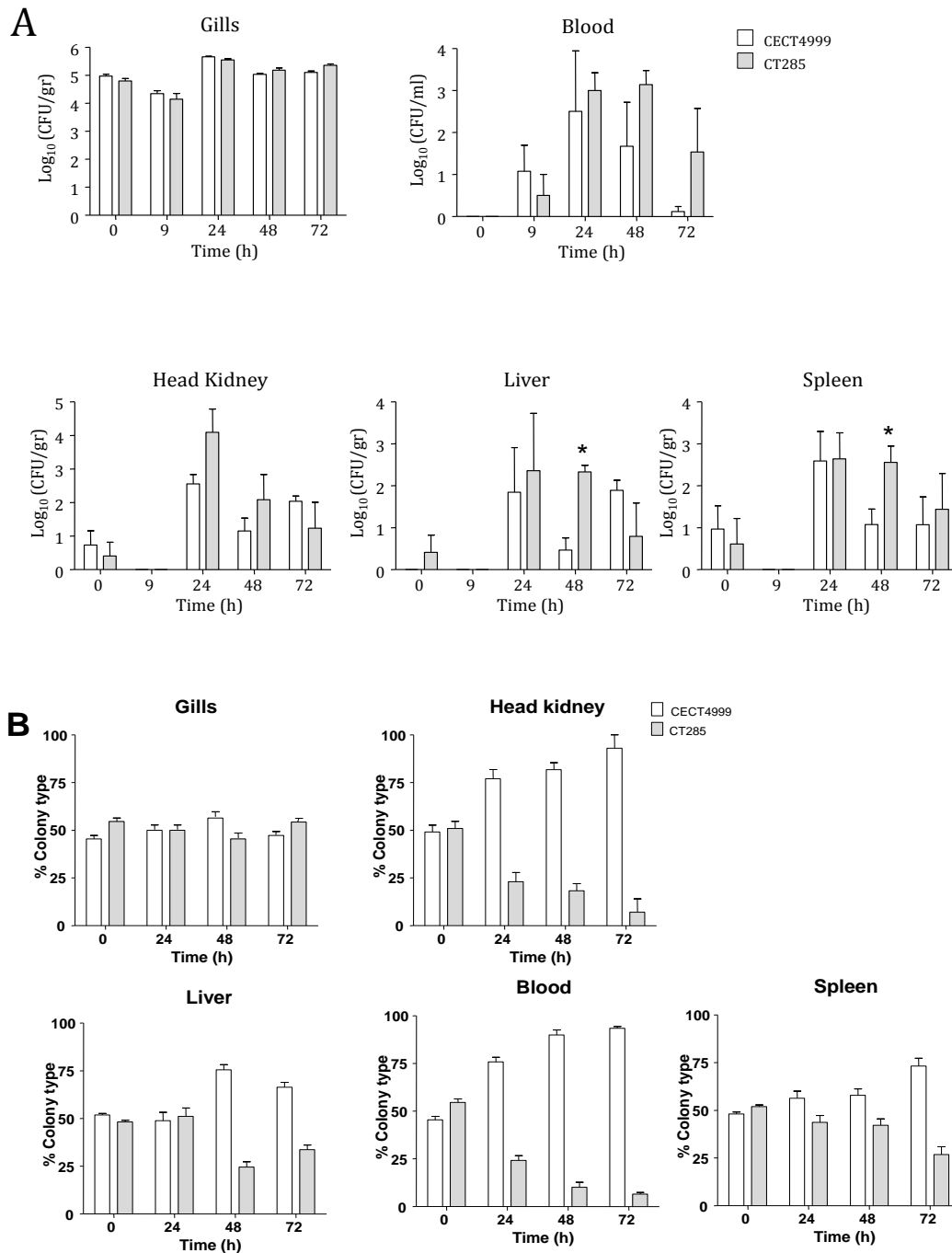
Figure 2. Eel colonization and invasion assays. A) <u>Infection experiments</u>: eels were infected by immersion challenge with the wild-type strain (CECT4999) or with the double mutant in *rtxA1₃* (CT285) and microbial counts on TSA-1 from external and internal organs were performed at different time intervals post-challenge. Asterisks indicate the significant differences (p<0.05) when compared with the wild-type strain. B) <u>Co-infection experiments</u>: eels were co-infected by immersion with strains CT285 and CECT4999 in a ratio 1:1 (vol:vol) at a dosis of 1.5x10⁶ CFU/ml and the percentage of each strain recovered on the plates is indicated on the Y-axis.

C.   Histopathology

The external and internal organs of the infected eels were examined histologically. In accordance with the low bacterial counts in internal organs, either an absence of bacteria or very few bacteria were observed in infected eel tissues by electron microscopy. Clinical signs were only observed in wild-type strain, while double mutant strain showed no alterations. Hemorrhaging was the only evident alteration observed in tissues of the eel challenged with the wild-type strain. Although hemolysis was not obvious, nonspecific changes, such as a slight alteration in the mitochondrial structure in the hematopoietic cells of head kidney or a mild increase in the number of phagocytosed erythrocytes in the spleen at 24 h and 48 h after challenge were observed (Figure 3A). Finally, the granulocytes were the main cell type that showed clear signs of damage (Figure 3B-D). Granulocyte damage was observed very early in kidneys (at 1 h post-challenge) and later (from 9 h post infection) in the head kidney, the main hematopoietic tissue in fish, and was mainly evidenced by release of cytoplasmic content, including granules (Figure 3B-D).
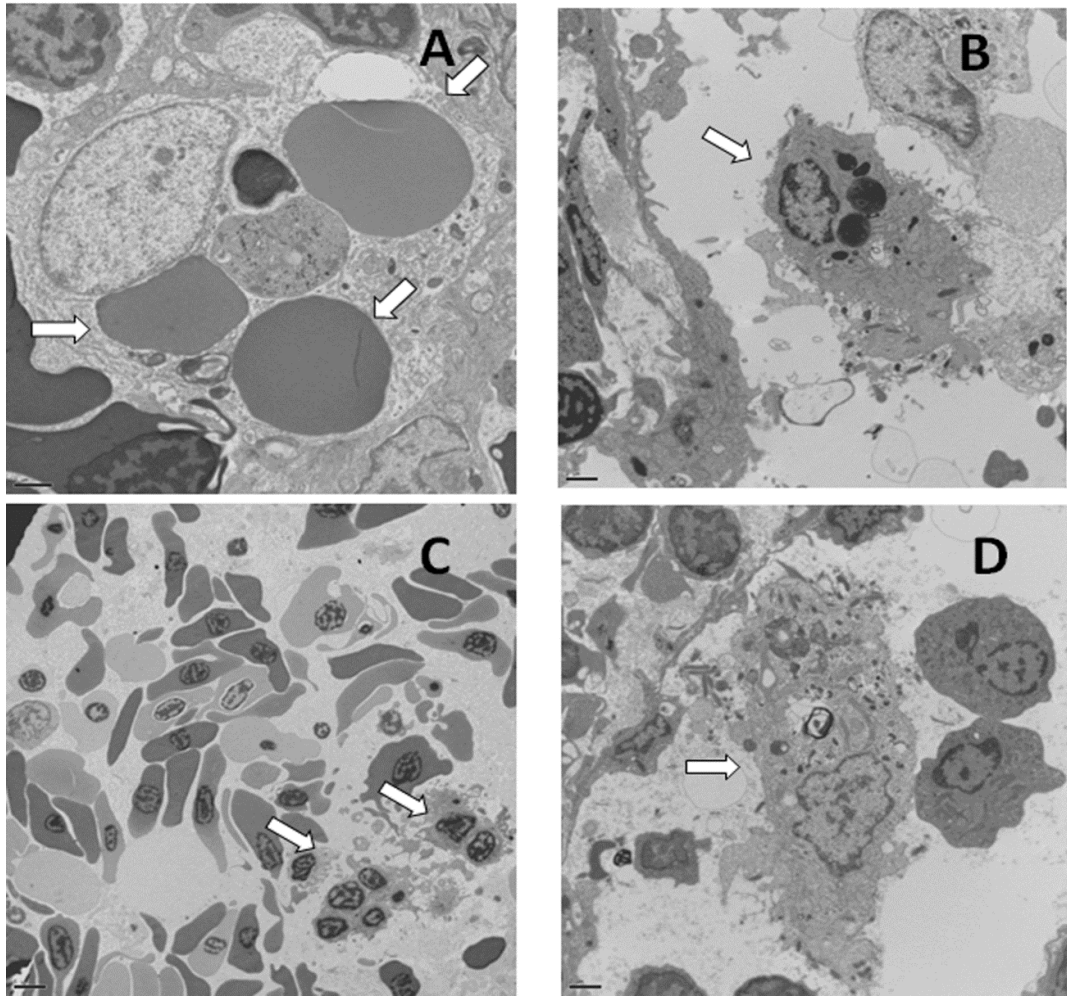
Figure 3. Histological analysis of the eels infected with the wild-type strain CECT4999. A) Macrophage with damaged erythrocytes (marked with arrows) engulfed within its cytoplasm. Bar, 1 µm. B-D) Three images of head kidney showing damaged granulocytes (marked with an arrow): B) bar, 1 µm; C) bar, 5 µm, and D) bar, 2 µm.

D.   Cytotoxicity for host cells

We determined cytotoxicity of the wild-type strain, mutant and complemented strains to epithelial fish and human cell lines as to freshly isolated eel and human erythrocytes. The wild-type strain proved to be toxic towards the three cell lines tested (Figure 4). Mutants lacking one copy of *rtxA1₋₃* exhibited wild-type cytotoxicity levels, while mutants lacking both copies of *rtxA1₋₃* showed significant reductions in cytotoxicity levels in relation to the wild-type strain (Figure 4). No significant differences were observed in the cytopathic effects of the complemented strains compared with the wild-type strain (Figure 4A and B).

Regarding eel and human erythrocytes, significant differences were found in hemolysis between the double mutant and the wild-type/double complemented strains (Figure 5A and B). Interestingly, the double mutant became immotile and aggregated in presence of wild-eel erythrocytes (Figure 5C). Bacterial aggregation was not observed when the double mutant was incubated with cultured-eel or human erythrocytes (data not shown).

In all cases, significant differences in lytic activity in all the tested cell types between double mutant and wild-type/complemented strains were only noticeable up until 1.5 h of incubation (Figure 5 and data not shown) after which all strains underwent complete cell lysis.
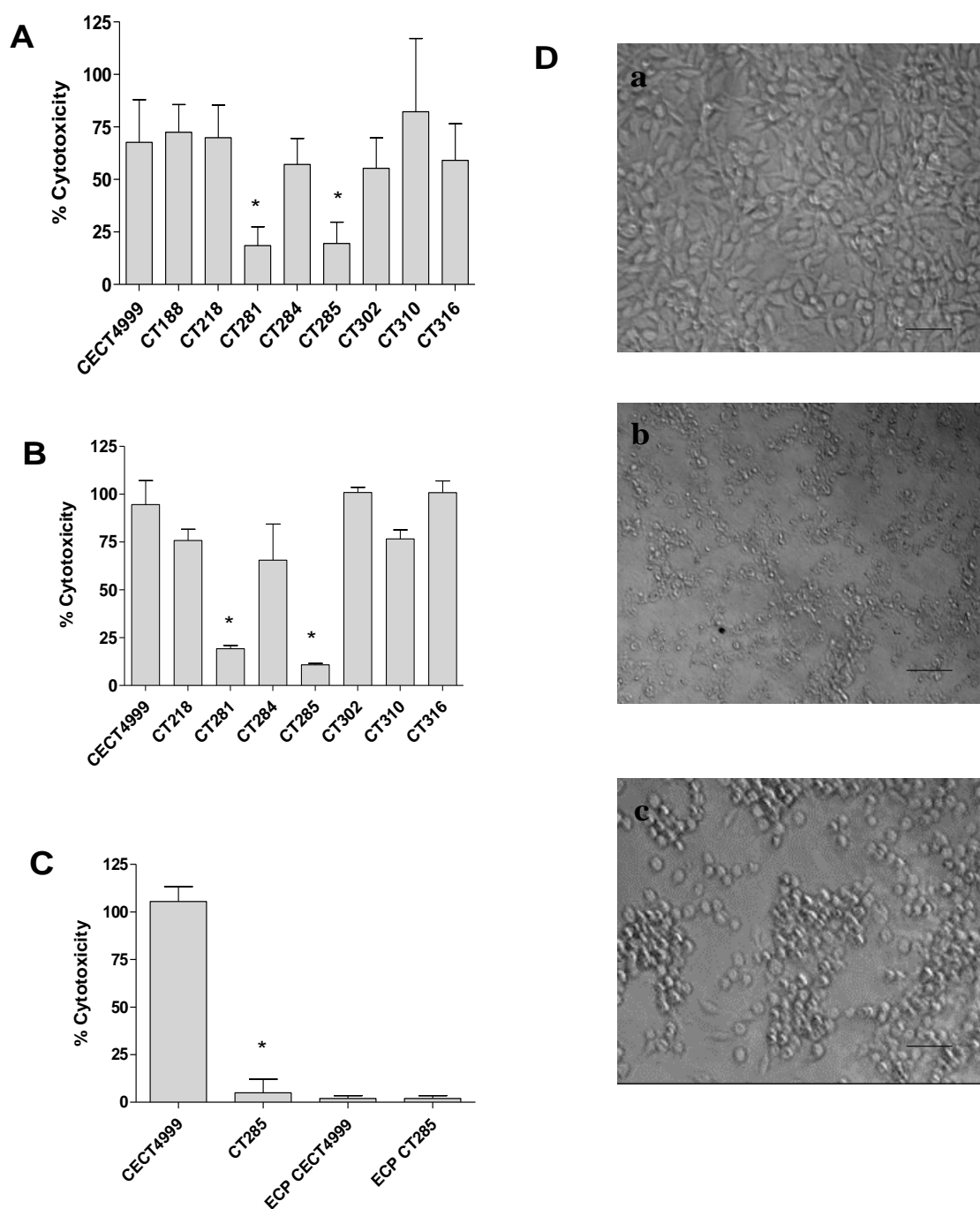
Figure 4. Cytotoxicity of different *V. vulnificus* biotype 2 strains to EP-1, HEp-2 and EPC cells. Cytotoxicity of bacteria or ECPs was determined by the released LDH through measuring the absorbance of the reaction mixture at 490 nm for EP-1 (A), HEp-2 (B) and EPC (C) cell lines. Asterisks indicate the significant differences ($p < 0.05$) when compared with the wild-type strain. The data were from an average of three independent experiments and were taken at time 90 min. D) Microscopic observation of EPC cells inoculated with *V. vulnificus* ECPs; a, control; b, ECP from CECT4999; c, ECP from CT285. Bar, 50 μm.
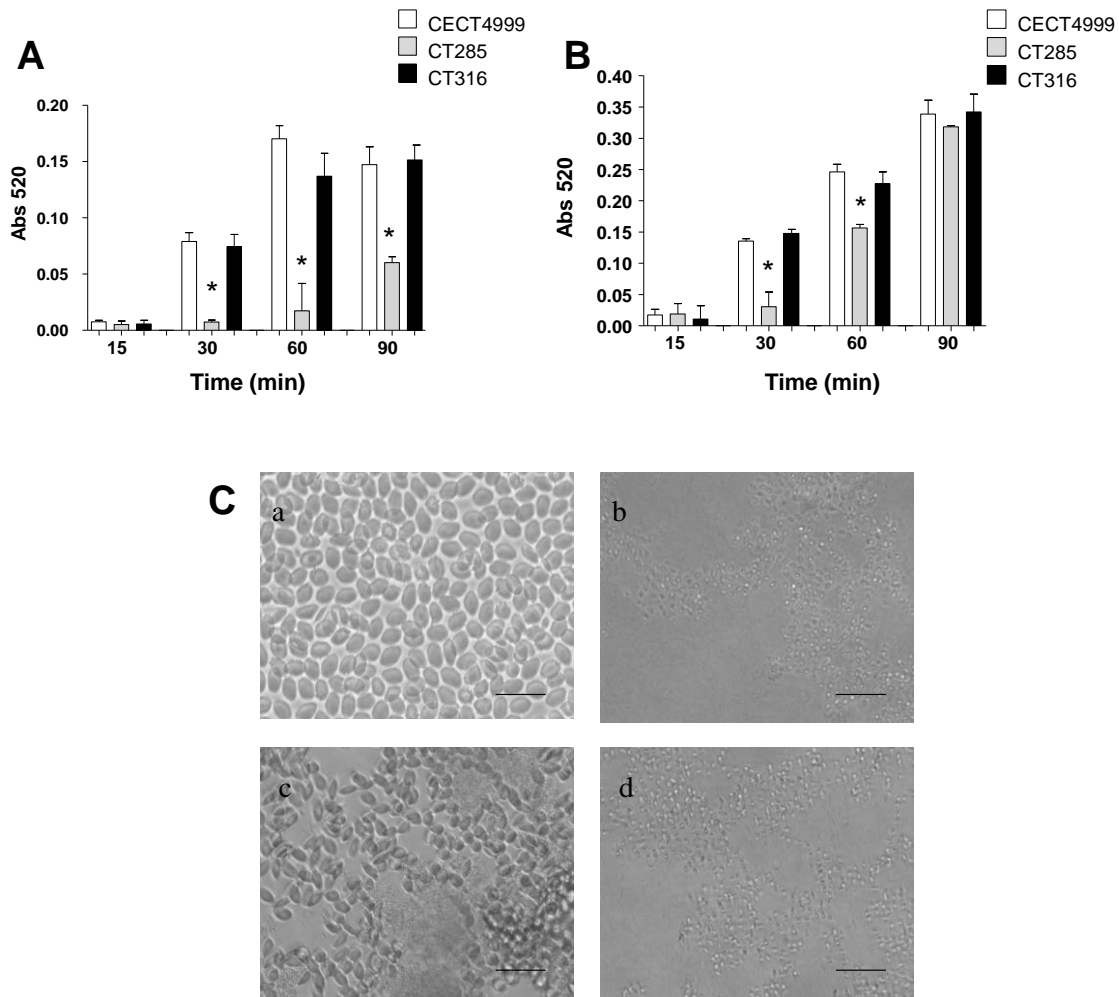
Figure 5. Cytotoxicity of different *V. vulnificus* biotype 2 to erythrocytes from eels and humans. The wild-type strain and its derivatives were incubated with eel (A) or human (B) erythrocytes in a 96-well plate and hemolysis was determined by measuring the OD_{520} of the supernatant at different time intervals. Asterisks indicate the significant differences ($p < 0.05$) when compared with the wild-type strain. The data were from an average of three independent experiments. C) Microscopic observation of wild-eel erythrocytes infected with CECT4999 (b), CT285 (c), and CT316 (d) at 60 min post-infection. Control (a), non-infected wild-eel erythrocytes. Bar, 100 μm.

Finally, no significant differences were observed in the cytopathic effects produced by the ECP of any of the strains (Figure 4C), which confirmed the results obtained in the eel toxicity assays. In fact, cytopathic effects were observed within 1 h and were manifested by rounding, shrinking, dendritic elongation and, finally, cell detachment, but not by cell lysis (Figure 4D).

E.   Interaction with host innate immune system

1.   Growth in plasma

Resistance to the bactericidal effect of plasma was tested by growing the bacteria in CM9 supplemented with fresh eel plasma (EP) or human plasma (HP) for 4 h. No differences were found in terms of bacterial growth in plasma among the different strains, with the exception of the cured strain, which was sensitive to fresh EP (Table 1).

2.   Phagocytosis by professional phagocytes

To ascertain whether MARTX type III provides protection against phagocytosis, the strains were incubated in presence of eel PEC as well as murine macrophages. PEC obtained from eels were identified as a macrophages and monocytes, and were isolated in a high number that allowed working with a monolayer. Microscopic observations of PEC preparations revealed that they were enriched in neutrophils (Figure 6). As shown in Figure 6A and 6E, the wild-type strain and the double-complemented resisted phagocytosis by eel PEC; they were not internalized, multiplied extracellularly (bacterial counts between approx. 1×10^6 at time 0 and 1×10^7 CFU/well at 90 min of incubation) and destroyed the monolayer in less than 60 min. The double mutant also multiplied extracellularly to a similar extent (counts between approx. 1×10^6 at time 0 and 1×10^7 CFU/well at 90 min of incubation) but it was poorly phagocytosed (maximal efficiency of 1 per 10^4 bacteria) (Figure 6A) and did not destroy the monolayer within 60 min of incubation (Figure 6E). In addition, the internalized double mutant cells did not survive intracellularly, since they were destroyed by eel PEC within 90 min (Figure 6C). In contrast, the murine macrophages phagocytosed all the analyzed strains much more efficiently than eel PEC, specially the double mutant, which was internalized in significantly higher numbers than the other two strains (Figure 6B). In all cases, the internalized bacteria were also killed by the mouse macrophages (Figure 6D). These results suggest that MARTX type III could protect from engulfment by the phagocytes but not from the bacterial destruction mechanisms inside the phagocyte.
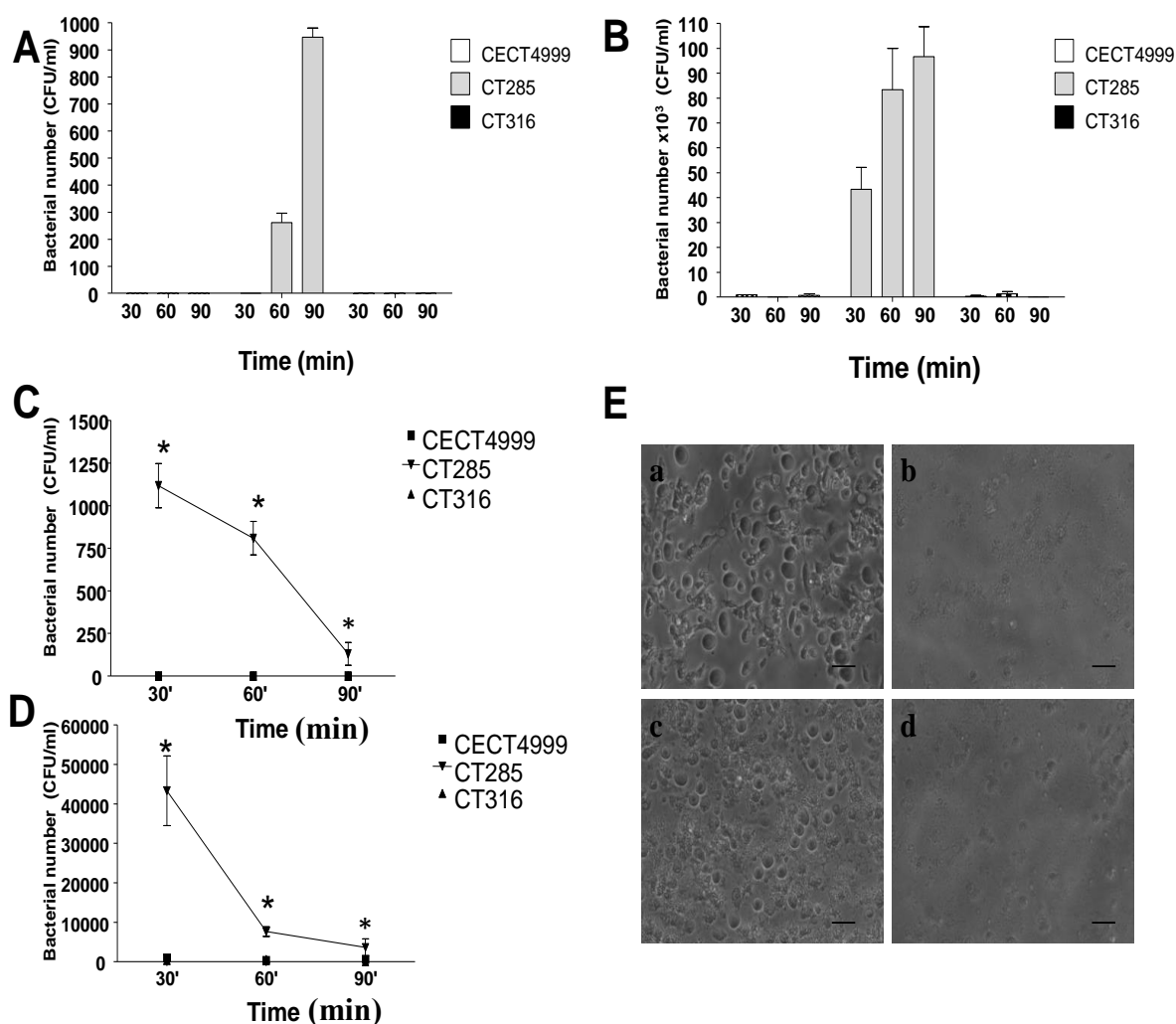
Figure 6. Interaction of different strains of *V. vulnificus* with phagocytes. Number of intracellular bacteria after 30, 60, and 90 min of incubation of CECT4999, CT285 and CT316 in monolayers of fresh eel PEC (A) or murine macrophages (RAW264.7) at a moi of 10. (B). Data were from an average of three independent experiments. Asterisks indicate significant differences (p<0.05) when compared with the wild-type strain. Survival inside eel PEC (C) or murine macrophages (D) after 30, 60 and 90 min was determined as bacterial counts as described in Material and Methods. The data were from an average of three independent experiments. Asterisks indicate the significant differences (p<0.05) when compared with the wild-type strain. E) Lysis of eel PEC produced by CECT4999 (b) and CT316 (d) but not by CT285 (c) at 60 min. post-infection. Control (a), non-inoculated eel PEC.

Samples of PEC infected with wild-type, double mutant and complemented strains, prepared as described above, were also stained with Hemacolor to visualize

directly if bacteria were phagocyted by the PEC. Results showed that, accordingly with the results obtained in phagocitosys assay, the wild-type and the complemented strain were not found inside PEC, but the double mutant strain was detected directly inside PEC (Figure 7).
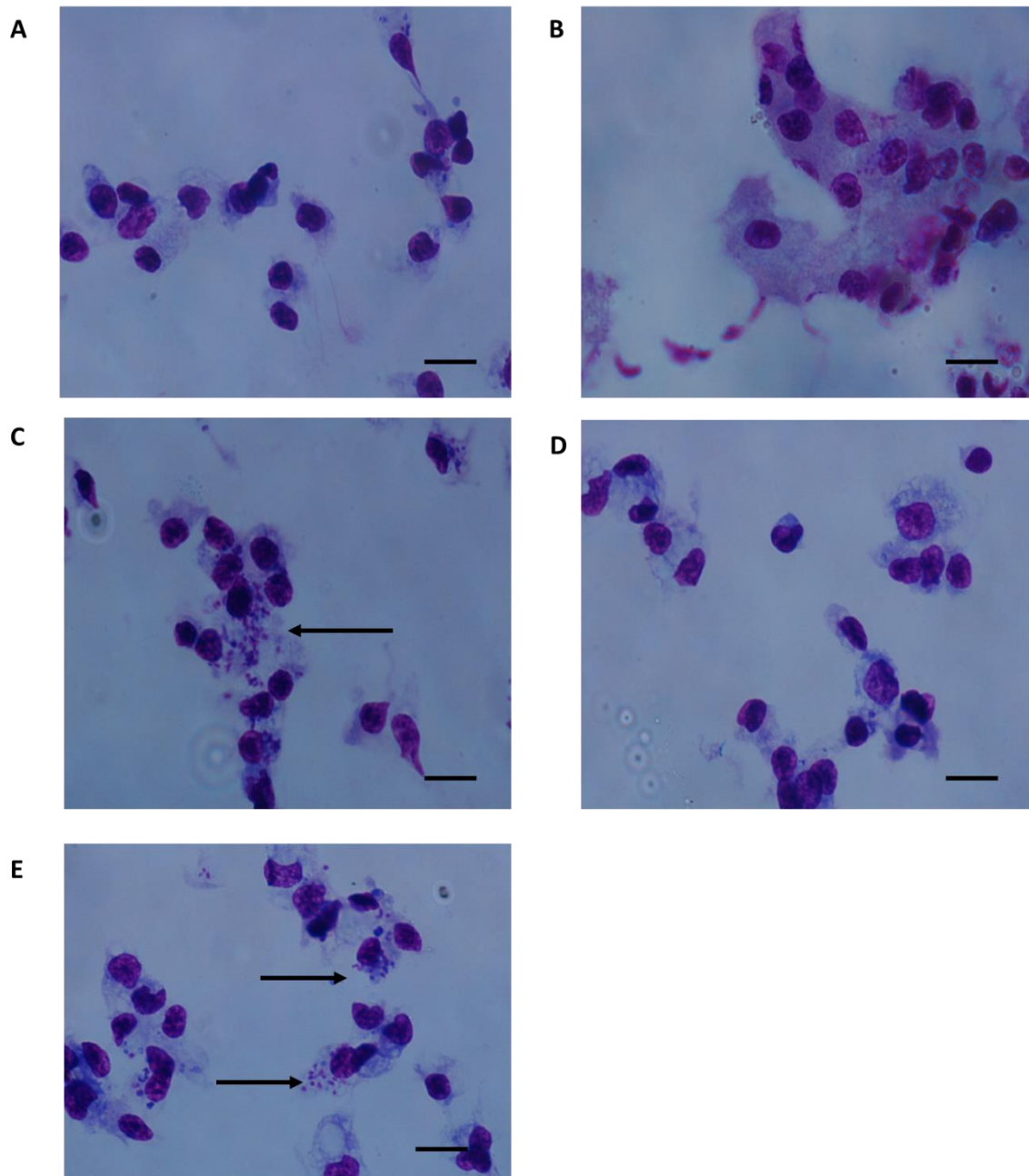
Figure 7. Hemacolor staining of PEC infected with *V. vulnificus*. A monolayer of PEC was infected with *V. vulnificus* at a moi of 10 and samples were stained at 60 min post-infection and stained with Hemacolor kit. A) Control. B) Wild-type strain C) and E) Double mutant strain. D) Complemented strain CT316.

F.   Amoeba predation

To ascertain whether MARTX type III may promote biotype 2 survival in the environment by destroying its putative natural predator, the amoeba, we isolated amoeba from the gills of different cultured fish species. Amoebae were successfully isolated and purified from turbot (*Scophthalmus maximus*) gills. These amoebae were identified using morphological (Leiro *et al.*, 1998) and phylogenetic criteria (Zhang *et al.*, 2000) as belonging to the species *Neoparamoeba pemaquidensis,* a gill disease-causing amoebic species. The amoebae were cultured with live bacteria from the wild-type or the double-mutant strain. As shown in Figure 8A, *N. pemaquidensis* started to grow exponentially from day 14 in the presence of the double mutant; however, the amoebae grew significantly less in the presence of the wild-type strain. In addition, destruction of amoebae, particularly during the first week of incubation, was observed when they were cultured with the wild-type strain but not with the double mutant (Figure 8B). This destruction seemed to be by cellular apoptosis (Figure 8Bc).
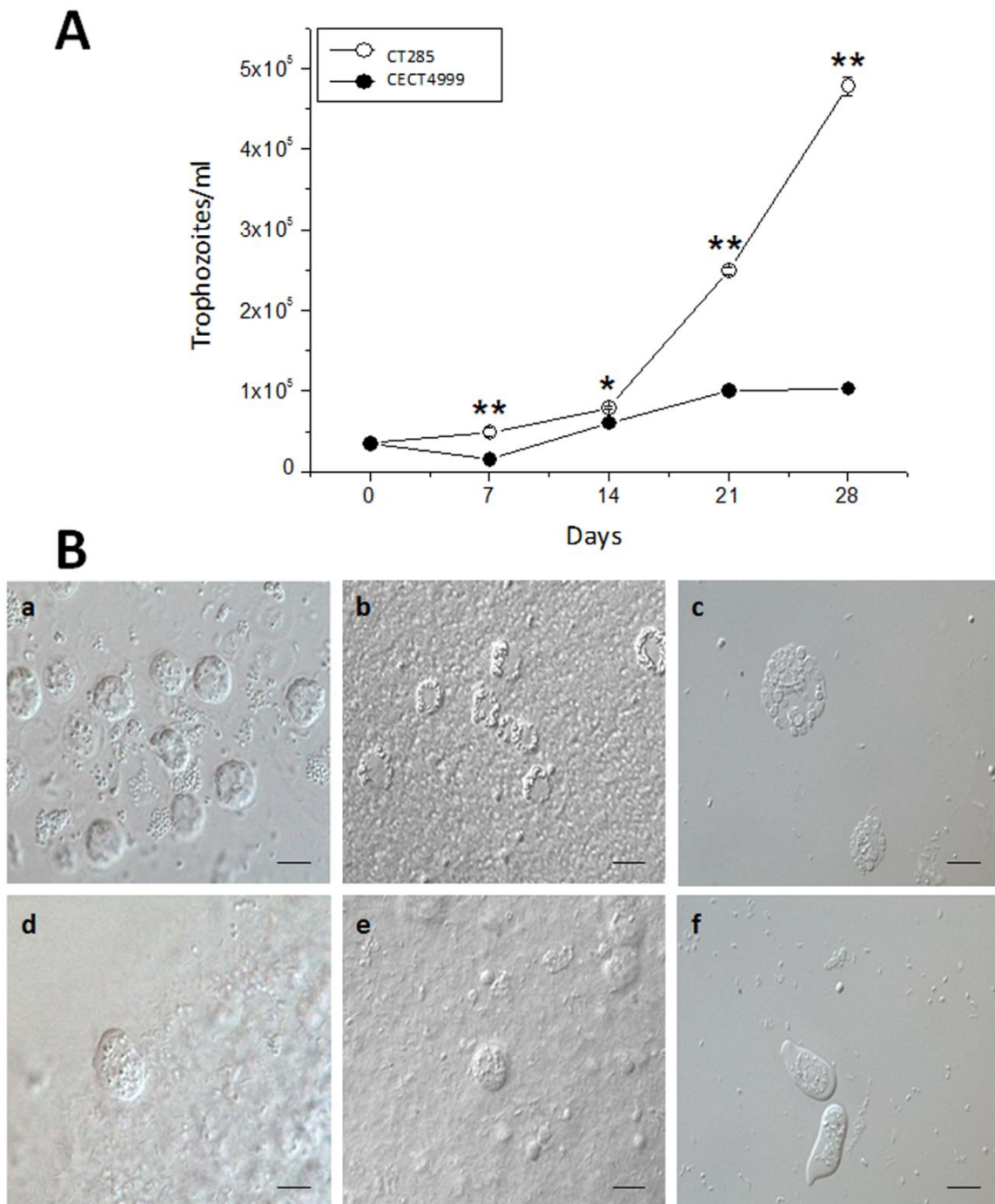
Figure 8. Interaction of different strains of *V. vulnificus* with amoeba. A) Growth curve of amoeba (*Neoparamoeba pemaquidensis*) incubated with CECT4999 or CT285. The data were from an average of three independent experiments. Asterisks indicate the significant differences (*, p<0.05: **, p<0.01). B) Differential interference contrast of amoeba grown with CECT4999 at time 0 (a) and 3 days (b and c) post incubation (notice that all amoeba are plasmolysed) or with CT285 at time 0 (d) and 3 days (e and f) post-incubation. Bar, 5 μm.

G.   Gene expression analysis of *rtxA1₃* gene

To determine the environmental cues involved in *rtxA1₃* expression, the transcriptional levels of *rtxA1₃* were assayed by qRT-PCR after growth under a variety of culture conditions mimicking the *in vivo* growth. As shown in Figure 9A, *rtxA1₃* expression in cultured bacteria was hardly affected by the presence (by adding ferric chloride, hemoglobin or hemin) or absence (by adding the iron-depleting compound, human apotransferrin) of iron in the culture media. However, *rtxA1₃* expression was increased 3- or 4-fold in the presence of either PEC or erythrocytes from eels or, even in the presence of amoeba (Figure 9B) but only if bacteria came into contact with eukaryotic cells (Figure 9C). A significant increase in *rtxA1₃* expression was also observed in infected eel blood at 9 h post-infection (Figure 9D). The transcriptional level of *rtxA1₃* declined to an undetectable level at 48 h post-infection, the time by which 50% of eels had died.
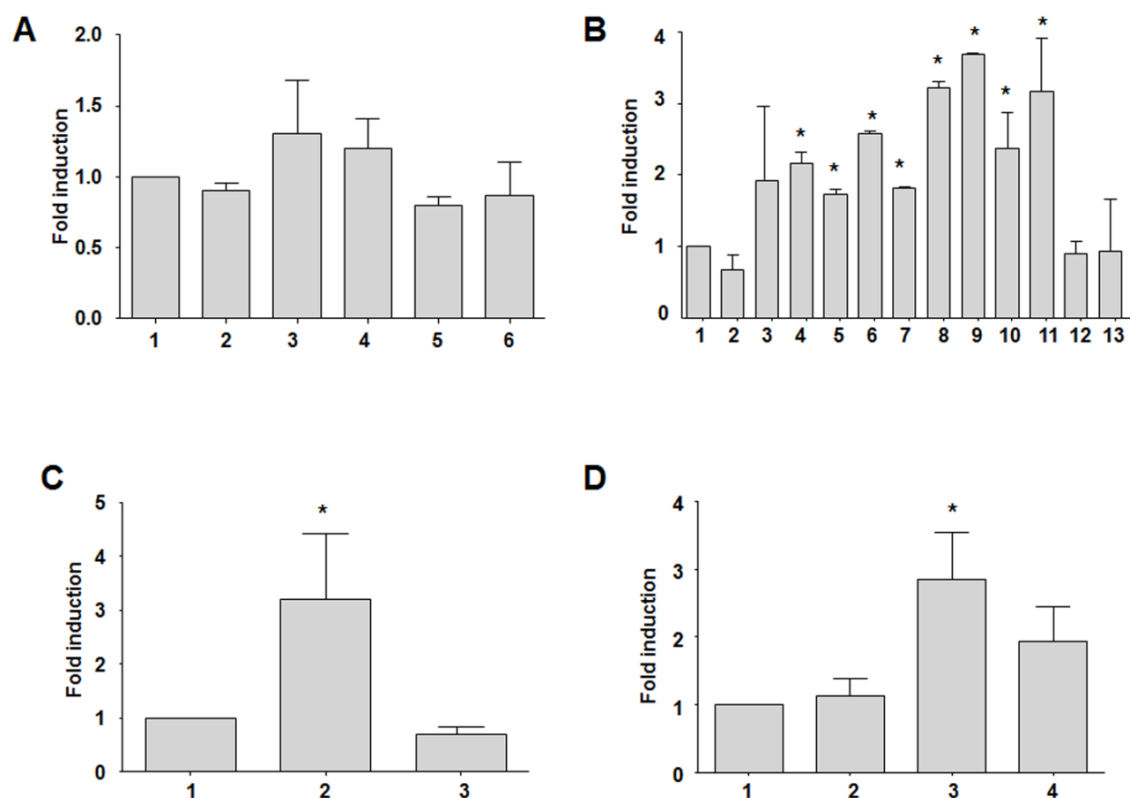
Figure 9. *rtxA1₃* **expression** *in vitro* **and** *in vivo* **and contact experiments**. Fold induction of *rtxA1₃* in strain CECT4999 growing *in vitro* and *in vivo*. A) **_In culture media_**: RNA was extracted from 1 ml of culture at mid-log phase point, which is indicated for each condition in parenthesis. 1, CM9 (6 h); 2, CM9-Fe (5 h); 3, CM9-Tf (9 h); 4, CM9-EP (8 h); 5, CM9-HP (7 h); 6, CM9-Hb (6h). B) **_In presence of eukaryotic cells_**: Bacteria were cultured in presence of eukaryotic cells maintained in L-15 (fish cells) or PBS (amoeba) at a moi of 10 and RNA was extracted from 1 ml of culture at different time intervals. Control L15 or PBS (1); L15 with eel phagocytes (EP) at 30 min (2), 60 min (3), 90 min (4) and 120 min (5) post-incubation; L15 with eel erythrocytes (EE) at 30 min (6), 60 min (7), 90 min (8) and 120 min (9); PBS with amoebae at 2h (10), 6h (11), 9h (12) and 24h post-incubation (13). C) **_Contact experiments_**. Bacteria were incubated with or without contact with EE at a moi of 100 and samples were taken after 3 h of incubation. (1), L-15; (2), with contact; (3), without contact. D) **_In vivo experiments_**. RNA samples were extracted from blood of immersion-infected eels after 0h (2), 9h (3) and 24h (4) post-challenge. Control (1): RNA from 1 ml of culture in CM9 at mid-log phase point. Asterisks indicate the significant differences ($p < 0.05$) when compared with bacteria grown in the respective control culture medium.

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IV. DISCUSSION

The study reported here has tested the hypothesis that MARTX type III is essential for *V. vulnificus* biotype 2 survival in the fish farming environment, in other words both inside and outside its main host, the eel. We selected a strain belonging to the zoonotic serovar with the aim of comparing the results obtained in the eel with those obtained in the mouse (the animal model used to predict virulence for humans). The results of virulence and *in vivo* expression assays clearly demonstrate that $rtxA1_3$ is a virulence gene, expressed in the internal tissues of eels during the infection process. In addition, $rtxA1_3$ also seems to be a virulence determinant in mice. However, the importance of $rtxA1_3$ in virulence is not the same in both animal models because inactivation of the two copies implies a complete loss of virulence for eels (increase in LD_{50} of more than 5 log. units) but only attenuated virulence for mice (increase in LD_{50} of two log. units). Another important difference is that only one copy of $rtxA1_3$ seems necessary for full virulence in eels while two copies are required for mice. Previous studies have also reported a two-log-unit attenuation in virulence for $rtxA1_1$ defective mutants in mice (Kim *et al.,* 2008; Lee *et al.,* 2007; Liu *et al.,* 2007; Lo *et al.,* 2011), suggesting that MARTX types I and III, although structurally different, could act similarly in a murine model of infection.

MARTX type I is recognized as a colonization and invasion factor for mice (Lo *et al.,* 2011). To ascertain whether $rtxA1_3$ mutants are avirulent because they are defective in eel colonization and invasion, *in vivo* colonization assays were performed by immersion. Contrary to that reported for Δ*rtxA1* mutants in mice, the double mutant in $rtxA1_3$ was not apparently deficient in either colonization or invasion in the eel. This mutant was able to attach to the gills and spread to the blood and to the internal organs, where it survived for at least 72 h in numbers that did not differ significantly from those reached by the wild-type strain. Nevertheless, we cannot discount the possibility that the toxin increases the survival rate in blood and head kidney because the double mutant was recovered in a lower proportion than the wild-type strain in the co-infection experiments. Regarding the clinical signs, the double-mutant infected animals did not show any apparent external or internal sign and survived throughout the experimental period. In contrast, the eels infected with

the wild-type strain died in the expected proportion (50%) within 72 h showing external and internal hemorrhaging, which would suggest that MARTX acts as a lethal factor for fish.

To ascertain what underlay the toxic effect caused by MARTX type III, tissues taken from wild-type and double mutant infected animals were microscopically analyzed and compared. The only cell alteration that could be clearly linked with MARTX type III was cell damage and release of cytoplasmic content, including granules of granulocytes (a class of cells that includes neutrophils), mainly from the hematopoietic tissues. Indirect evidence of alterations affecting erythrocytes was also observed.

To test the hypothesis that the target for MARTX type III *in vivo* might be the granulocytes and, secondarily, the erythrocytes, cytotoxicity experiments were performed with freshly isolated eel erythrocytes and PEC. In contrast to that measured in the bacteria grown in different iron-depleted culture media and plasma, transcription of *rtxA1₃* was up-regulated when the bacteria were co-cultured with both cell types. In both cases, the wild-type and the double complemented strains lysed a significant proportion of eel PEC and erythrocytes within 90 min while the double mutant was unable to do so. Consistent with this result, none of the wild-type bacteria was phagocytosed while the double mutant was phagocytosed, albeit poorly, by eel PEC, a finding that is compatible with this strain's ability to colonize and invade the eel. Our results also suggest that MARTX type III could lyse the epithelial cells from fish and mammals, as observed with MARTX type I (Kim *et al.*, 2008; Liu *et al.*, 2007; Lo *et al.*, 2011).

It has been reported that MARTX type I exerts its activity only upon bacteria-eukaryotic cell contact (Kim *et al.*, 2008). To test whether cell contact is also required for MARTX type III cytotoxicity, we evaluated *rtxA1₃* expression in presence of eel erythrocytes by separating them, or not, with a 0.22 µm-pore filter. The results indicate that expression of MARTX type III, like MARTX type I, requires bacterium-eukaryotic cell contact.

Interestingly, the double mutant agglutinated in the presence of eel erythrocytes from wild eels. This result suggests that eel erythrocytes secrete some

anti-bacterial component (possibly an agglutinin) that may be involved in the defense against vibriosis. Recently, Morera *et al.* (2011) described an active role of salmonid erythrocytes against pathogens. According to the results of this work, an active role of erythrocytes against pathogens could be extended to eel erythrocytes. In contrast, no bacterial aggregation was observed with erythrocytes from cultured eels, which correlates with the general immunodepressed state that eels manifest under captivity (R. Barrera, personal communication). As expected, no bacterial aggregation was visualized in the presence of human erythrocytes, which are non-nucleated cells.

Contrarily to that observed in the eel, murine macrophages were able to phagocytose the wild-type bacteria, although less efficiently than the double mutant, and all internalized bacteria were killed by 90 min. Similar results were obtained by Lo *et al.* (2011) and suggest that MARTX types I and III, although structurally different, could act in the same way in mice by protecting the bacteria from phagocytosis.

Rapid eel death without gross clinical signs after being infected with the wild-type strain is congruent with previous studies suggesting that the eels died from peracute septic shock. Biosca and Amaro (1996) clearly demonstrated that LPS of *V. vulnificus* is not one of the toxic factors involved in septic shock in eels. In fact, most fish species lack orthologs for Toll-like receptor 4, the specific receptor for LPS in mammals (Iliev *et al.,* 2005). The results obtained in this work suggest that MARTX type III could be the main toxic factor triggering this septic shock in fish infected with biotype 2. The transcriptome of immunostimulated eels has recently been sequenced (Callol *et al.*, 2011) and the genome of CECT4999 is being annotated (unpublished results). Further studies into the host-pathogen interactions at the transcriptomic level are underway to validate this hypothesis.

The presence of *rtxA1$_3$* gene in duplicate was confirmed in all the analyzed strains of our *V. vulnificus* biotype 2 collection, regardless of clonal origin, serology or virulence degree for eels (Roig *et al.*, MS in preparation). It is not clear why this gene varies in structure and is duplicated in *V. vulnificus* biotype 2 strains. In fact, possession of this gene does not provide a clear evolutionary advantage to the bacterium since this work shows that MARTX type III triggers overly rapid animal

death, without giving the bacterium time to multiply or reach similar population sizes to other fish pathogenic vibrios (Lamas *et al.,* 1994). To test whether MARTX type III could confer survival advantages to the bacterium outside the host, we isolated fish amoeba from turbot gills and cultured it in the presence of the wild-type strain or the double mutant. We observed destruction of amoebae by the wild-type strain, but not by the double mutant, and detected up-regulation of the *rtxA1₃* gene in presence of fish amoeba. This indicating that MARTX type III could be involved in bacterial resistance to amoebal predation. Interestingly, the amoebal destruction microscopically resembled to that derived from cellular apoptosis, finding that has to be confirmed in further studies. *V. vulnificus* biotype 2 survives after antibiotic treatment in farms by forming biofilms on the fish surface, mainly on the gills (Marco-Noales *et al.*, 2001). The results of the present study suggest that MARTX type III could be used by the bacterium in the biofilms to increase its survival rate in the fish farming environment.

In conclusion, MARTX type III of *V. vulnificus* biotype 2 seems to be involved in the interaction of this organism with a wide range of eukaryotic cells, ranging from amoebae to professional phagocytes. In any event, after bacterium-cell contact this toxin seems to cause cell lysis by an unknown mechanism. While in the mouse MARTX type III seems to act as a colonization factor preventing the bacterial cells from phagocytosis, it may function as a toxin involved in the onset of septic shock in the eel. Furthermore, this toxin may promote *V. vulnificus* biotype 2 survival in the environment by killing the amoeba, putative predator of this organism, which is a plausible explanation for the wide distribution of the *rtx* gene cluster among different clones of this polyphyletic group.

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CHAPTER 2

IRON ACQUISITION SYSTEMS OF THE ZOONOTIC SEROVAR OF *Vibrio vulnificus*

I.   INTRODUCTION

Nutritional immunity, the most ancient system of defense against pathogens common to all vertebrates (Weinberg E. D., 2009), consists of metabolic adjustments in order to make iron unavailable to microorganisms. To overcome iron-starvation in host tissues, *V. vulnificus* Bt1 produces two siderophores: vulnibactin (a catechol) and an unnamed hydroxamate siderophore (Okujo *et al.,* 1994; Simpson and Oliver, 1983). The Bt1 seems to use vulnibactin as the main siderophore for scavenging iron from human transferrin both *in vitro* and *in vivo*. Thus, Bt1 mutants deficient in vulnibactin production or in the vulnibactin receptor (VuuA) grow less efficiently in iron-deficient media and are attenuated in mice virulence (Kim *et al.,* 2006; Litwin *et al.,* 1996; Webster *et al.,* 2000). In addition, *V. vulnificus* Bt1 can utilize non-Tf-bound iron through a heme receptor, HupA (Litwin *et al.,* 1998) also involved in virulence for mice (Oh *et al.,* 2009). Recently, a novel heme-specific receptor without any known role in virulence, HutR, has been described in *V. vulnificus* Bt1 (Datta and Crosa, 2012). *V. vulnificus* Bt2 seems to produce phenolates and hydroxamates and use hemin (Hm) as the sole iron source (Biosca *et al.,* 1996; Fouz *et al.,* 1996). The chemical nature of the siderophores as well as the role of iron-acquisition systems in virulence of the zoonotic variant is unknown. Moreover, *V. vulnificus* Bt2 possesses a virulence plasmid (pVvBt2) that confers resistance to the eel innate immune system (Lee *et al.*, 2008; Valiente *et al.*, 2008a). This plasmid contains an ORF, Vep20, that presents similarity to a transferrin binding protein, which could be involved in specific virulence for fish.

The present study is focused on the host-nonspecific and -specific iron acquisition systems used by the zoonotic serovar to infect humans and fish. These systems are usually under Fur control. As a first approach, we identified the iron-uptake genes by using the Fur titration assay (FURTA) that enables identification of Fur-regulated genes (Stojiljkovic *et al.,* 1994) and, subsequently, we obtained single and multiple mutants by allelic exchange in selected genes of the strain CECT4999. The mutants and the wild-type strain were used in a series of *in vitro* and *in vivo* tests including virulence for eels and mice, animals models for fish and human vibriosis, respectively. Finally, the evolutionary history of the identified virulence

genes was inferred and compared with that of the species by multilocus sequence analysis (MLSA).

II.   MATERIALS AND METHODS

A.   Bacterial strains and growth conditions

1.   General growth conditions

Bacterial strains (Table 1) were routinely grown in LB-1/LBA-1 (Luria-Bertani broth/agar, 1% NaCl) or in CM9/CM9A (Sambrook and Russel, 2001) at 28ºC (*V. vulnificus*) or 37ºC (*E. coli*) and were stored in LB-1 plus glycerol (17%) at -80ºC. For FURTA (see later), the bacterial strains were grown on McConkey agar base (Difco) supplemented with 1% lactose and 0.04 mM $FeSO_4$ (M+Fe). If necessary, ampicilin (100 µg/ml) or polymixin B (50 U/ml) were added to the media.

2.   Growth in artificial media supplemented with different iron sources

Fresh eel blood, and erythrocytes and plasma from eel and human were obtained as described in Chapter 1, page 78. Bacteria were grown in CM9-HP (CM9 + fresh human plasma in proportion 1:1 [vol/vol]), CM9-EP (CM9 + fresh eel plasma in proportion 1:1 [vol/vol]), CM9-HP-Fe20/200 (CM9-HP + 20 or 200µM $FeCl_3$), CM9-EP-Fe20/200 (CM9-EP + 20 or 200 µM $FeCl_3$) and CM9-EE (CM9 + 1% eel erythrocytes in PBS + 100 µM ethylenediamine-di-[o-hydroxyphenylacetic] acid [EDDHA; Sigma]). Bacteria were also grown in CM9A-E (CM9 agar + 100 µM EDDHA), CM9-Fe (CM9 + 100 µM $FeCl_3$), CM9-Hm-0.1/10 (0.1 or 10 µM bovine Hm [Sigma] + 100 µM EDDHA), CM9-Hb-10 (10 µM bovine hemoglobin [Sigma] + 100 µM EDDHA) and CM9-Tf (40 µM iron-free human apo-Transferrin [Sigma]).

Table 1. Strains and plasmids used in this study.

Designation	Description	Isolation source/ Reference
V. vulnificus		
529[T]	Biotype 1	Human blood (USA)[a][b]
YJ016	Biotype 1	Human blood (Taiwan)[c][b]
CS9133	Biotype 1	Human blood (South Korea)[b]
B2	Biotype 1	Human blood (China)[b]
MO24/06	Biotype 1	Human blood (South Korea)[b]
CMCP6	Biotype 1	Human blood (South Korea)[b]
94-8-119	Biotype 1	Human wound (Denmark)[b]
E64MW	Biotype 1	Human wound (USA)[b]
CG100	Biotype 1	Oyster (Taiwan)[b]
JY1305	Biotype 1	Oyster (USA)[b]
JY1701	Biotype 1	Oyster (USA)[b]
CECT4608	Biotype 1	Healthy eel (Spain)[b]
CECT4866	Biotype 2 Serovar E	Human blood (Australia)[b]
CIP8190	Biotype 2 Serovar E	Human blood (France)[b]
94-8-112	Biotype 2 Serovar E	Human wound (Denmark)[b]
CECT5763	Biotype 2 Serovar E	Eel tank water (Spain)[b]
CECT4604	Biotype 2 Serovar E	Diseased eel (Spain)[b]
CECT4999	Biotype 2 Serovar E	Diseased eel (Spain)[b]
CECT5198	Biotype 2 Serovar A	Diseased eel (Spain)[b]
CECT5768	Biotype 2 Serovar A	Diseased eel (Spain)[b]
CECT5769	Biotype 2 Serovar A	Diseased eel (Spain)[b]
A11	Biotype 2 Serovar A	Diseased eel (Spain)[b]
A13	Biotype 2 Serovar A	Diseased eel (Spain)[b]
95-8-7	Biotype 2 Serovar I	Diseased eel (Denmark)[b]
95-8-6	Biotype 2 Serovar I	Diseased eel (Denmark)[b]
95-8-161	Biotype 2 Serovar I	Diseased eel (Denmark)[b]
95-8-162	Biotype 2 Serovar I	Diseased eel (Denmark)[b]
11028	Biotype 3	Human blood (Israel)[b]
12	Biotype 3	Human blood (Israel)[b]
Δ*vep20*	CECT4999 *vep20*-defective mutant	This study
Δ*hupA*	CECT4999 *hupA*-defective mutant	This study
Δ*vuuA*	CECT4999 *vuuA*-defective mutant	This study
Δ*hutR*	CECT4999 *hutR*-defective mutant	This study
Δ*hupA*Δ*vuuA*	CECT4999 *hupA vuuA*-defective double mutant	This study
Δ*hupA*Δ*hutR*	CECT4999 *hupA hutR*-defective double mutant	This study
c*vep20*	Δ*vep20* complemented strain	This study
c*hupA*	Δ*hupA* complemented strain	This study
c*vuuA*	Δ*vuuA* complemented strain	This study
E. coli		
DH5α	Cloning strain	Invitrogen
H1717	*araD139 ΔlacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR aroB fhuF::λ placMu*	Hantke, 1997
s17-1λ*pir*	Strain containing the pCVD442 plasmid. *thi pro hsdR hsdM+ recA::RP4-2-Tc::Mu λpir Km[r] Nal[r]*	Simon *et al.* (1983)
Plasmids		
pUC18	Cloning vector Amp[r]	Fermentas
pCVD442	Suicide vector; *sacB* , *bla*, mobRP4 and R6k ori	Donnenberg *et al.*(1991)
pGemT-easy	T/A Cloning vector, Amp[r]	Promega
pIT009	Derivative of pJRD215 with the Sm[r] gene between two *Xmn*I sites replaced by the multiple-cloning-site-containing *lacZ* gene cloned from pUC19	Lee *et al.* (2008)
pΔ*vep20*	pCVD442 with Δ*vep20* in the MCS	This study
pΔ*hupA*	pCVD442 with Δ*hupA* in the MCS	This study
pΔ*vuuA*	pCVD442 with Δ*vuuA* in the MCS	This study
pΔ*hutR*	pCVD442 with Δ*hutR* in the MCS	This study
pIT*vep20*	pIT009 with *vep20* gene and promoter in MCS	This study
pIT*hupA*	pIT009 with *hupA* gene and promoter in MCS	This study
pIT*vuuA*	pIT009 with *vuuA* gene and promoter in MCS	This study

[a] Type strain of the species
[b] Strains whose published sequences were used for the MLSA: sequences for *vvha, rtxA1, wzz*, *pilF*, *glp*, *mdh, pyrC*, and *pntA* were taken from Chen *et al.* (2003), Kim *et al.* (2003), Sanjuan *et al.* (2011), Morrison *et al.,* (2012), Wang *et al.* (2012) and Roig *et al.* (2011).
[c] Strain used as reference for primer design for genes *vuuA*, *hupA* and *hutR*.

B.   DNA/RNA manipulation

1.   General technics

The general techniques of acid nucleic manipulation, PCR and qRT-PCR are detailed in Chapter 1, pages 71-74. All primers were designed from the sequences of *V. vulnificus* YJ016 and CECT4999 (Table 2).

2.   Fur Titration Assay (FURTA)

FURTA is based on multiple plasmid-encoded Fur boxes derepressing chromosomal Fur-regulated genes by titrating the Fur protein (Stojiljkovic *et al.,* 1994). FURTA was performed according to Osorio *et al.* (Osorio *et al.,* 2004). Total DNA from *V. vulnificus* CECT4999 strain was extracted and partially digested by using the frequent cut restriction enzyme *Sau*3AI, and the 0.5-6 kb fragments were cloned in the *Bam*HI site of the multicopy plasmid pT7-7. The obtained library was transformed into in *E. coli* H1717 by electroporation on M+Fe. This strain carries a Fur-regulated *fhuF::lacZ* gene fusion. When a multicopy plasmid containing a Fur-regulated promoter is introduced in this strain, cause the de-repression of the fusion by titrating the Fur protein, thus leading to transcription of the *lacZ* gene and the expression of a Lac+ phenotype, that in M+Fe were identified as red transformants.

3.   Isolation of mutant and complemented strains

Single and multiple in-frame mutants were obtained by allelic exchange (Shao and Hor, 2000). Briefly, a series of plasmids were created in pCVD442 (a suicide vector that allows negative selection by sucrose) (Donnenberg and Kaper,

1991) by cloning fragments that contained the up-stream and downstream region of each gene with an in-frame deletion of the major part of the coding sequence (Table 1). The plasmids pΔ*vep20*, pΔ*hupA*, pΔ*vuuA* or pΔ*hutR*, containing the up- and downstream regions of *vep20, hupA, vuuA* and *hutR,* respectively, were transferred by conjugation from *Escherichia coli* S17-1λ*pir* (Table 1) to strain wild-type CECT4999 to get single mutants. To obtain double mutants, the corresponding plasmids were transferred by conjugation to the corresponding single mutants (Table 1). Transconjugants were subsequently selected with 10% sucrose from those that have lost pCVD442 via second homologous recombination event. Complemented strains c*vep20,* c*hupA* and c*vuuA* were generated by conjugal transfer of the wild-type genes, obtained with primers vep20-cF/vep20-cR, hupA-cF/hupA-cR or vuuA-cF/vuuA-cR (Table 2), cloned in pIT009 (Lee *et al.,* 2008) (pIT*vep20*, pIT*hupA* and pIT*vuuA)* (Table 1).

Table 2. Primers used in this study.

Primer	Restriction site	Sequence	Product size (bp)	Utilization
vep20-1	*Sac*I	GTGAGCTCTACTGGTCAAAG	1302	Mutant construction
vep20-2	*Hin*dIII	GGAAGCTTCCCAAAGAAGTACCTCGAAC		Mutant construction
vep20-3	*Hin*dIII	CGAAGCTTGCGACCCTGTCCTGTTCG	1278	Mutant construction
vep20-4	*Xba*I	CGTCTAGACCTCTGGCTGTAATTGC		Mutant construction
hupA-1	*Sph*I	CGGCATGCCAGTAAGAATCCATTAGAGG	1401	Mutant construction
hupA-2	*Kpn*I	CGGGTACCCGTGATTTAACTCAAGCAG		Mutant construction
hupA-3	*Kpn*I	CGGGTACCATCTTGAGCTTGTACTGG	1407	Mutant construction
hupA-4	*Sph*I	CGGCATGCGTCCTGATGAATAAGATC		Mutant construction
vuuA-1	*Sal*I	CGGTCGACATTCCTACACTTAGCCGC	1404	Mutant construction
vuuA-2	*Kpn*I	CGGGTACCCTAAAACAGCAACCACGT		Mutant construction
vuuA-3	*Kpn*I	CGGGTACCCCCCATCACTACCGCAGAC	1401	Mutant construction
vuuA-4	*Sac*I	CGGAGCTCTCCGTGATGATATTGCTAAG		Mutant construction
hutR-1	*Sal*I	GCGTCGACTATGCCGCCAGTGATGCAAA	1435	Mutant construction
hutR-2	*Pst*I	GCCTGCAGGTTGGCAGCGAGTACCGAC		Mutant construction
hutR-3	*Pst*I	GCCTGCAGACTTATTCCACAGAGCCGGGG	1423	Mutant construction
hutR-4	*Sph*I	GCGCATGCCCATACATACCTTGCAAAACG		Mutant construction
vep20-cF	*Xba*I	CTTCTAGACGAGCAAATATGCCATGC	3180	Mutant complementation
vep20-cR	*Xba*I	GGTCTAGAGCATCTTCAATCGCTAACGG		Mutant complementation
hupA-cF	*Bam*HI	TTAGAAGTTGTATTTCACAC	2366	Mutant complementation
hupA-cR	*Bam*HI	TTTAACTCCTTTGGTGATC		Mutant complementation
vuuA-cF	*Xba*I	CTAGAAGTTCAACTGCAATG	2407	Mutant complementation
vuuA-cR	*Xba*I	AGGCATCTCATGCGGTGAG		Mutant complementation
vep20-seq1F		GTGACACTAGAGTGCCTGAA	718	Sequencing
vep20-seq1R		AGGATCTTGCTTGGTCGGT		Sequencing
vep20-seq2F		ATCATACCATGGGTTAGGC	679	Sequencing
vep20-seq2R		ATACGACCGTTCTCAAGACC		Sequencing
vep20-seq3F		AATCAATGTTTGCGTAAACG	707	Sequencing
vep20-seq3R		CTGTCAATATTAACAAAGGG		Sequencing
vep20-seq4F		CACTCGCCTCTTTGGTTTCG	585	Sequencing
vep20-seq4R		GTTTGATATAATCCGTACG		Sequencing
hupA-seq1		GAATGAGACTTAAAAAGCC	1001	Sequencing
hupA-seq2		CCTGATGCGAAGGAAATGA		Sequencing
hupA-seq3		TCATAACGAACACCAGGAG	964	Sequencing
hupA-seq4		CAGCCAGGCGTGTTTGAT		Sequencing
hupA-seq5		CATATCCGGATCAACCGTGA	500	Sequencing
hupA-seq6		GGAACGACATAAGAGCCAT		Sequencing
vuuA-seq1		CTCTGGTCAACATCAGAGGC	1122	Sequencing
vuuA-seq2		ATGATCGATACACTAATCCG		Sequencing
vuuA-seq3		AACTCTTTACCTTCAGTGG	1101	Sequencing
vuuA-seq4		CATCCTGAATGCAATCAG		Sequencing
hutR seq-1		GGACAGGCGTAAAGGATTGG	1229	Sequencing
hutR seq-2		GACGCTCAGACGTTCTCGAA		Sequencing

hutR seq-3	TGCTGATATGACCAAGGCG	1231	Sequencing
hutR seq-4	TGCTGTACTTGCTCGACGC		Sequencing
recA-F	CGCCAAAGGCAGAAATCG	59	qRT-PCR
recA-R	ACGAGCTTGAAGACCCATGTG		qRT-PCR
vep20-qF	CACTCGCCTCTTTGGTTTCG	72	qRT-PCR
vep20-qR	GGGACTGATTCTCTCTTC		qRT-PCR
hutR-qF	CATGGCGGATGTTGAAGATATC	76	qRT-PCR
hutR-qR	AACTGCGTTTTTGCTCCGTAA		qRT-PCR
hupA-qF	AAGCTAGATGCTGCGCCTTT	60	qRT-PCR
hupA-qR	CACGGTTGATCCGGATATGC		qRT-PCR
vuuA-qF	GGACCACGGGAATCCATATG	56	qRT-PCR
vuuA-qR	TGCGTTGGCGGGTTTTA		qRT-PCR
Plug-F	ATGAAAAGTTTATTATTTAT	441	Recombinant protein
Plug-R	TGCACCACCTAAACTACCGG		Recombinant protein
TonB-F	GTGTCTTACGAAACCAAAGAGG	1782	Recombinant protein
TonB-R	CTATAACTTAACTTCAAGTCC		Recombinant protein

C.   Phenotypic characterization of mutants

1.   Outer membrane proteins (OMPs)

To relate the genes *vep20*, *vuuA*, *hupA* and *hutR* with its corresponding OMP, the wild-type strain and its single mutants were grown in CM9-Fe and CM9-Tf for 12 h, and then OMP were extracted as described previously (Biosca *et al.,* 1993). OMP samples were fractionated by sodium dodecyl sulfathe-polyacrilamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) by using a separation gel of 10 % acrylamide. The protein bands were stained with Coomassie brilliant blue.

2.   Siderophore detection

The Chrome azurol S (CAS) assay was used to detect the siderophore production in iron-restricted growth cultures (Schwyn and Neilands, 1987). The Arnow phenolic acid assay and the Csàky hydroxylamine hidroxamic acid assay were carried out to detect catechol- and hydroxamic-type siderophores, respectively, as previously described (Arnow, 1937; Andrus *et al.,* 1983). The strains *V. anguillarum* RV22 and *Photobacterium damselae* ssp *damselae* CECT626[T], were

used as positive controls for Arnow and Csàky tests, respectively (Biosca *et al.,* 1996).

3. Growth in hemin and transferrin

The ability of the wild-type and Δ*hupA*, c*hupA,* Δ*hutR* and Δ*hupA*Δ*hutR* strains to use Hm as the sole iron source was tested by measuring bacterial growth (OD_{600}) in CM9-Hm at 1-h intervals during 10 h (Mouriño *et al.,* 2004). 96-well plates were inoculated with an overnight culture in CM9 (1:100, vol/vol) and were incubated at 28ºC with shaking (200 rpm).

The ability of the wild type, Δ*vep20,* Δ*vuuA* and c*vuuA* strains to use iron from iron-saturated human Tf (holo-Tf, Sigma) was assayed as Simpson and Oliver (Simpson and Oliver, 1987) by measuring the growth halo around Tf-discs (soaked in a solution of holo-Tf 1mM) placed on CM9A-E plates previously inoculated with 100 µl of an overnight culture in CM9, and by inoculating the CM9-Tf medium with an overnight culture in CM9 (1:100, vol/vol).

4. Growth in plasma and blood

To simulate the *in vivo* growth in host bloodstream, bacteria were grown as described in Chapter 1, page 78, in whole blood or plasma. The bactericidal (complement) or bacteriostatic (Tf) activity of plasma was abolished by heating it at 56ºC for 30 min (Amaro *et al*., 1997) or supplementing it with 100 µM of $FeCl_3$, respectively.

5. Phagocytosis

The ability of bacteria to escape from the phagocytic activity of phagocytes was assayed as described in chapter 1 page 78. If necessary, bacteria were opsonized as described by Valiente and cols. (Valiente *et al*., 2008b); briefly, 900 µl of a bacterial suspension on HBSS containing 10^9 CFU/ml was mixed with 100 of

previously heat-inactivated eel plasma, incubated 1 h at room temperature and washed with HBSS.

6. Cell damage (erythrocytes)

Eel erythrocytes were collected from blood by centrifugation (3000 rpm, 15 min, 4°C), washed three times with HBSS (Hank's balanced salt solution), resuspended in L-15 and distributed in a 96-well plate in a concentration of 10^5 cells per well. The plate was centrifuged to spin down cells. The monolayer of erythrocytes was infected with L15-washed bacteria (harvested from a 4 h culture in L-15) at a moi of 10, and microscopic observations were made at 60-90 min post-infection with a Nickon optic microscope.

7. Bacterial attachment

To determine the ability of the wild-type strain and its mutants to attach to dry blood, 96-well plates coated with air-dried eel-blood were inoculated with 100 μl of a bacterial suspension of 10^9 CFU/ml in PBS-1 from 1 ml of overnight culture in CM9-Tf, and were incubated at room temperature for 24 h. Bacterial DNA was obtained at 0, 3, 9 and 24 h post-incubation after washing wells twice with PBS and lysing bacteria by adding Mili-Q water. DNA was quantified by quantitative PCR (qPCR) by using primers specific for *recA* housekeeping gene (recA-F/recA-R) (Table 2).

8. Virulence and colonization

Animals were maintained as described in Chapter 1, page 80. The virulence degree for eels and mice was determined as explained in Chapter 1, page 80. In the case of mice, the animals were pre-injected with iron (Hm [2.8 μg/gr of mouse], $FeCl_3$, [9 μg/gr of mouse] or Hm+$FeCl_3$ [1.4 μg of Hm/gr of mouse + 4.5 μg of $FeCl_3$/gr of mouse]) 2 h before challenge. For colonization and invasion assays, eels were bath infected as detailed in Chapter 1, page 81, with either the wild-type strain or each

one of the single mutant and expressed as CFU/ml (blood) or CFU/g (gills, liver, kidney and spleen).

D.   Sequence analysis

1.   DNA sequencing

Amplicons were examined by agarose gel electrophoresis (1%) and ethidium bromide staining. PCR products of the predicted size were purified from agarose gel by using the High Pure PCR purification Kit (Roche) following manufacturer's instructions and sequenced in an ABI 3730 sequencer (Applied Biosystems).

2.   Phylogenetic analysis

The evolutionary scenario of *vuuA* and *hupA* was evaluated from the whole sequence of each gene and was compared with a MLSA reconstruction (Didelot and Falush, 2007) from the partial sequences (254 nt by gene) of four virulence-associated (*vvha, rtxA1, wzz*, and *pilF*) and four housekeeping (*glp*, *mdh, pyrC*, and *pntA*) genes taken from the Genebank (Chen *et al.,* 2003; Morrison *et al.,* 2012; Kim *et al.,* 2003; Roig *et al.,* 2011; Sanjuán *et al.,* 2011; Wang *et al.,* 2012). Phylogenetic trees for each single gene and for the concatenaded-MLSA were obtained using the maximum-likelihood method with PhyML software (Guindon *et al.,* 2009). The best evolutionary model for the sequences according to jModelTest (Posada, 2008) and considering the Akaike information criterion (AIC) (Akaike, 1974) turned out to be the Tamura 3-parameter model (Tamura, 1992) (T92) for the *vuuA* and *hupA* genes and for the MLSA-concatenate alignment. The model was applied with a gamma distribution and invariant sites accounting for heterogeneity in evolutionary rates among sites. Support for the groupings derived in these reconstructions was evaluated by bootstrapping using 1.000 replicates. No outgroups were used for the analysis of both genes due the enormous differences among species. The congruence among phylogenetic reconstructions obtained with the different alignments was checked using Shimodaira-Hasegawa (SH) (Shimodaira and Hasegawa, 1999) and

expected-likelihood weight (ELW) tests as implemented in TreePuzzle, version 5.2 (Schmidt *et al.,* 2002; Strimmer and Rambaut, 2002).

3. Molecular clock estimation of *hupA* and *vuuA*

The following equation was used to roughly determine the age of divergence for each pairwise comparison: number of synonymous SNPs (sSNPs)/(number of sSNP sites X mutation rate X number of generations per year) (Foster *et al.,* 2009; Galloway-Peña *et al.,* 2012). The sSNPs were selected because supposedly they are neutral or nearly neutral in terms of selection and therefore allow for a relatively unbiased estimation of SNP accumulation (Foster *et al.,* 2009). The number of potential sSNP sites for each codon was calculated from a lookup table of codon possibilities and added together to give the number of potential synonymous SNP sites for all the codons in the sequence. Since the synonymous mutation rate of *V. vulnificus* is not known, we selected a value of 1.4×10^{-10} mutations per base pair per generation based on mutation rates data from *Escherichia coli* (Lenski *et al.,* 2003). The generation time *in vitro* for *V. vulnificus* biotypes 1, 2 and 3 is 4.0, 2.9, and 2.4 generations h^{-1}, respectively (Chase and Harwood, 2011). However, there are no data on the generation time in the environment. On the basis of the estimations performed for *E.coli* (Ochman *et al.,* 1999) (100 to 300 generations/year), *Bacillus anthracis* (Van Ert *et al.,* 2007) (43 generations per year) and *Vibrio parahaemolyticus* (García *et al.,* 2012) (100 generations per year), we choosen a value of 365 generations per year for *V. vulnificus*.

E.   Analysis of Vep20 protein

1.   Bioinformatic analysis

Bioinformatic approaches were carried out to study the protein sequence and functionality of Vep20. Online programs PsortB (Yu *et al.,* 2010) for cellular location, SecretomeP (Dyrløv Bendtsen *et al.,* 2005) for signals of peptide secretion and pFam (Punta *et al.,* 2012) for functional domains were used.

2. Purification and expression of recombinant Vep20

The DNA regions corresponding to the plug domain (aminoacids nº 1 to 147) and the TonB-dependent receptor (TonB-dr) domain (aminoacids nº 148 to 741) of Vep20 protein were amplified by PCR with primers Plug-F/R and TonB-F/R, respectively. Amplicons were cloned in the vector pQE-30 (Qiagen) transformed in the expression strain *E. coli* M15 and His_6-tagged Plug and TonB-dr domains were induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified by using a nickel affinity column (NiNTA) as instructed by the manufacturer (Qiagen). A 20 ml bacterial culture, in which recombinant plug and TonB-dr were induced, was pelleted, resuspended in a lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, pH8), sonicated (6 pulses of 10 seconds at 100V) and centrifuged at 13000 rpm for 30 min at 4ºC. The supernatant was recovered and used as the soluble protein fraction, and for insoluble protein fraction obtention the protocol was the same but adding Urea 8M to the lysis buffer.

3. Immunization and antibody titer

Policlonal antibodies against Plug and TonB-dr domains were obtained in AntibodyBCN by immunization of eight-week-old specific pathogen-free BALB/c female mice (Antibody BCN, Spain). The dose was 50 μg of pure antigen combined with complete Freund's adjuvant and the rest of dosis were 50 μg combined with incomplete Freund's adjuvant in a total of 5 dosis every 15 days. Sera were collected before every intramuscular immunization for measurement of anti-Plug and anti-TonB-dr antibody titers, that were measured from immunized and control mice by ELISA as follows; *V. vulnificus* overnight cultures in CM9-Tf were pelleted and resuspended in PBS plus 1% dithiothreitol (DTT) and 1% sodium dodecyl sulfate (SDS), and incubated at 100ºC for 5 min. After centrifuge at 13000 rpm for 5 min, pellet was discarded and the supernatant was quantified by the Pierce BCA Protein Assay Kit (Thermo Scientific). The ELISA plates were coated with 5 μg of antigen-containing supernatant in 50 μl of Coating buffer (sodium carbonate 60 mM, pH 9.6) and incubated overnight at 4ºC. Wells were washed with wash buffer (PBS plus 0,05% Tween20 [Sigma]) and blocked with 100 μl of Blocking buffer (PBS plus 1%

bovine serum albumin [BSA][Sigma]) for 1 h at RT. Wells were washed three times and mice sera, diluted in Assay buffer (eBioscience), was used as a primary antibody with an incubation of 2 h at 37ºC. Wells were washed three times and the peroxidase-conjugated goat anti-mouse IgG was diluted in Assay buffer and used as the secondary antibody by incubating 1 h at RT. Finally, after seven washing steps, the antibody titers were determined by measuring the Abs_{450} after addition of TMB (3,3', 5,5'-tetramethylbenzidine, eBioscience) with a plate Multiskan EX.

4. Western blot

For each tested condition, 5 µg of protein, quantified with the Pierce BCA Protein Assay Kit (Thermo Scientific), were separated by Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) in discontinuous gels (4% stacking gel, 10% separating gel). Proteins were transferred from gel to a polyvinylidene difluoride (PVDF) membrane (0.2 µm, Bio-Rad) at 100V for 1 h in Tris-glycine-methanol transfer buffer (25 mM Tris, 192 mM glycine [pH 8.3], 20% [vol/vol] methanol) as described by Towbin and cols. (Towbin *et al.*, 1979). Immunostaining was performed with mice Vep20-specific primary antibody diluted at 1:3000 and anti-rabbit IgG HRP-conjugated diluted at 1:5000 (Sigma), and revealed with Immobilon Western Chemiluminescent HRP Substrate (Millipore) in an Agfa Curix 60 revelator.

F. Statistical analysis

All the experiments were performed by triplicate and the significance of the differences was tested by using the unpaired Student's t-test with a $P < 0.05$.

III. RESULTS

A. FURTA and preliminar characterization of selected genes

1. FURTA

The assay was performed as a first approximation to the identification of the Fur-regulated genes in the selected zoonotic strain CECT4999. The ORFs with significant homology to *V. vulnificus* Bt1 genes identified by FURTA are shown in Table 3. The set includes chromosomal genes such as the genes for the receptors HupA (clone DP006) and VuuA (clone DP009) (but not for the receptor HutR), for vulnibactin and heme transport and for vulnibactin biosynthesis as well as a plasmid gene that presents low homology to a putative transferrin-receptor (clone DP020) (lee *et al.* 2008). No gene related to hydroxamate-type siderophore biosynthesis could be identified although a cluster of genes for exogenous aerobactin utilization was found (Table 3). Accordingly, the strain was positive in the CAS assay, a universal assay for siderophore detection, positive in the test for phenolates and negative in the test for hydroxamates (Table 3).

Table 3. ORF's contained in FURTA positive clones of *V. vulnificus* CECT4999 with homology to ORF's of sequenced strains of *V. vulnificus*.

Clone	Features	Strain	GI number	% Indentity[1]
DP002	ABC-type hydroxamate-dependent iron transport system, ATPase	YJ016	37201709	95
DP004	NRPS	MO6-24O	319934358	95
	3-deoxy-7-phosphoheptulonate synthase		319934359	
DP006	Heme receptor HupA precursor (*hupA*)	MO6-24O	319933927	99
DP009	Ferric vulnibactin outer membrane receptor (*vuuA*)	YJ016	37201513	97
	Vulnibactin synthetase, amide synthase subunit		37201514	
DP010	Phosphomannomutase	YJ016	37201320	97
DP015	Vulnibactin 2,3-dihydroxybenzoate-2,3-dehydrogenase	CMCP6	27358808	97
DP020	Transferrin-binding protein A precursor	pR99	152955030	100
DP021	Hypothetical protein VV2_1010	MO6-24O	319934562	97
DP023	Ferritin-like protein 2	CMCP6	27360656	99
DP025	Ferric aerobactin receptor (*iutA*)	CMCP6	319999718	97
DP027	Flp pilus assembly protein CpaB	YJ016	37198942	88
DP033	Methionine aminopeptidase	YJ016	37199491	95
	PII uridylyl-transferase		37199492	
DP055	Vulnibactin-specific 2,3-dihydroxybenzoate-AMP ligase	MO6-24O	319934367	96
	Aryl carrier domain		319934368	
DP073	Aryl carrier domain	MO6-24O	319934368	97
	Catechol siderophore ABC transporter, substrate-binding protein		319934369	

[1] Percentage of identity in aminoacid sequence obtained by BLAST-P algorithm.

2.   Furboxes and gene sequencing

hupA and *vuuA*, together with *hutR* (selected despite not being identified by FURTA) were sequenced in the strain CECT4999 (Table 1) using primers from the genome sequence of the Bt1 strain YJ016 (Table 2). The sequences were deposited in the Genbank (KC741503, KC741545, KF056337). The genes *hupA, vuuA* and *hutR* showed 97%, 95% and 97% similarity values (in the amino acid sequence) with respect to the homologous ones in the Bt1 strain YJ016, respectively. *vep20* was re-sequenced in the strain CECT4999 using primers (Table 2) from the plasmid pR99 (Lee *et al.*, 2008) and the sequence obtained was identical to that previously published (Lee *et al.*, 2008).

Three furboxes in clones DP006, DP009 and DP020, containing a part of *hupA, vuuA* and *vep20* genes, were identified, GCTAATGATAATTACTATC, GCAAAGCATTCTCATTTGC and AATAATGATTATCATTATC, respectively, immediately upstream each one of the genes. The two first were highly similar to those reported by Litwin and Byrne (Litwin and Byrne*,* 1998) in *hupA* (identical) and Webster and Litwin (Webster and Litwin, 2000) in *vuuA* (18/19) while the third one was identical to that previously reported for this gene in the plasmid pR99 (Lee *et al.*, 2008).

3.   Transcription versus iron starvation

To relate each selected gene with iron-regulation, transcription level was assayed by growing the wild-type strain in presence and absence of iron. A positive fold-induction for the four genes was observed when bacteria were subjected to the iron-restricted conditions imposed by apo-Tf (Figure 1). In the case of genes for Hm receptors, the transcription of *hupA* was significantly higher than that of *hutR* (Figure 1). A positive fold induction of *vuuA*, *hupA* and *vep20* was also detected when fresh plasma from either humans or eels was added to CM9 (Figure 1). *vuuA* and *vep20* over-transcription were abolished when $FeCl_3$ was added to plasma at a concentration of 20 μM, while 200 μM were needed to abolish *hupA* over-transcription, which suggests that transcription of *vuuA* and *vep20* are more sensitive to iron concentration.
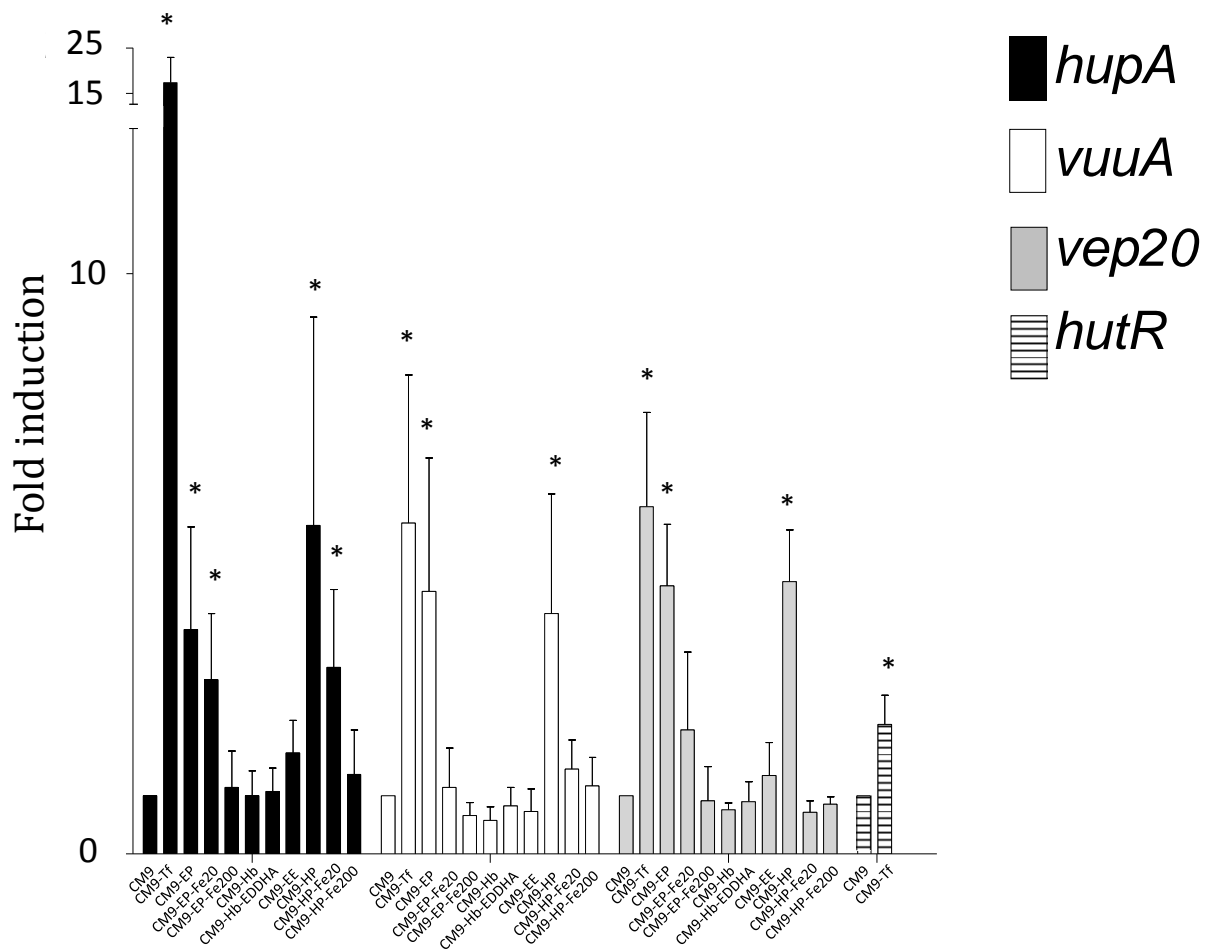
Figure 1. Analysis of gene transcription *in vitro* by qRT-PCR. Levels of mRNA were quantified in the mid-log phase of several conditions and expressed as fold induction. Asterisks indicate significant overexpression of each gene with respect to the expression level in CM9 ($p < 0.05$).

To relate growth rate and gene expression, the fold induction of *vep20*, *hupA* and *vuuA* vs growth of the wild-type strain in iron restriction conditions (apo-Tf or fresh eel plasma) was studied. As Figure 2 shows, the transcription of all genes was induced just before the early log-phase and was maintained for 10 h, indicating that the three genes are expressed before the utilization of Vep20, HupA and VuuA as iron receptors for active growth.
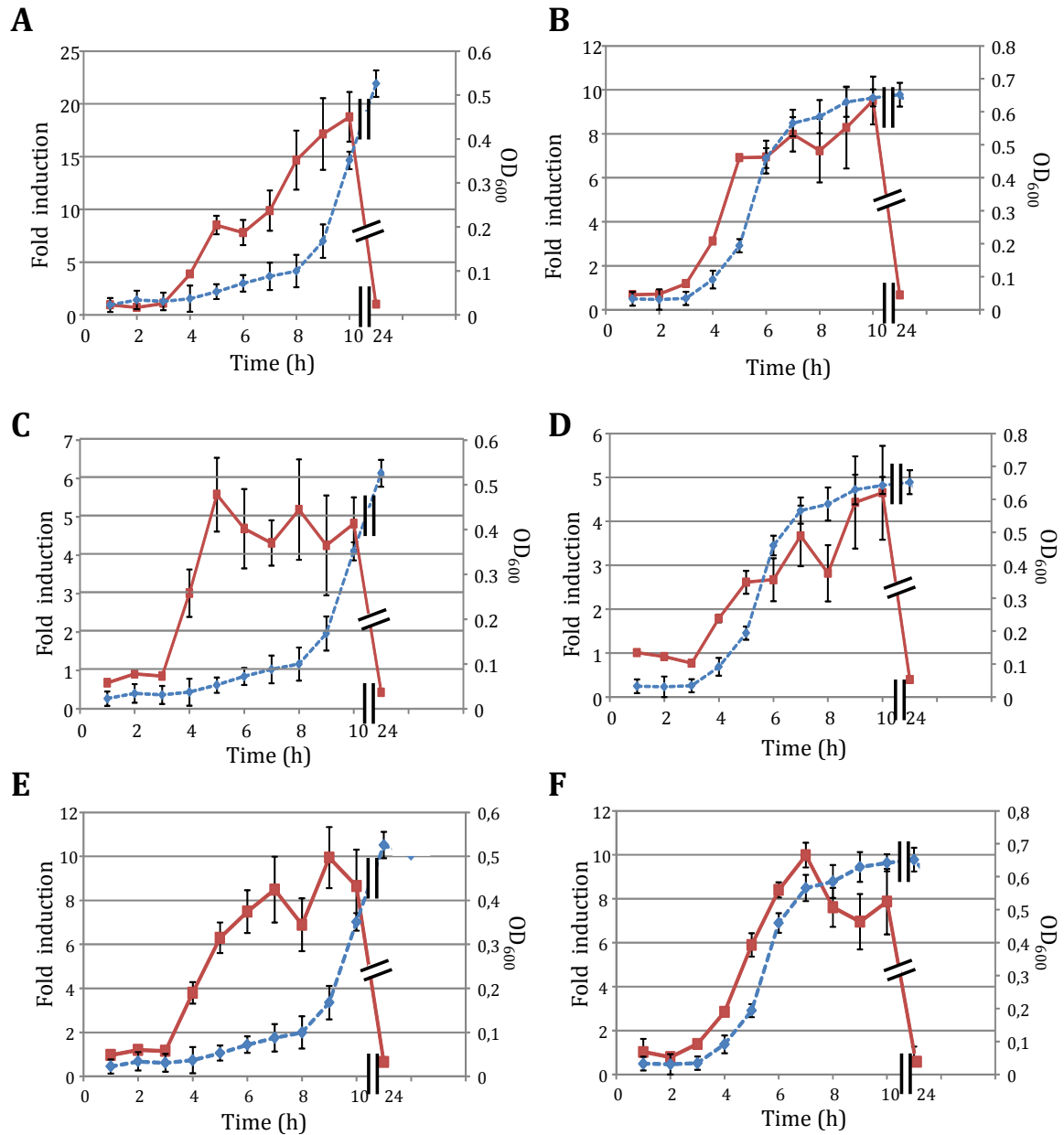
Figure 2. **Time course analysis**. Expression at transcription level of *hupA* (A, B), *vuuA* (C, D) and *vep20* (E, F) in CM9-Tf (A, C, E) and CM9-EP (B, D, F) measured by qRT-PCR (continuous line) vs bacterial growth (discontinuous line).

B.   Phenotypic characterization of mutants

1.   OMPs and siderophore production

According to Litwin and Byrne (Litwin and Byrne, 1998) and Webster and Litwin (Webster and Litwin, 2000) the OMP profiles of $\Delta hupA$ and $\Delta vuuA$ strains lack proteins of 77 and 72 KDa, respectively, which were present in the OMP profiles of the wild-type strain and the complemented strains (Figure 3). No difference in protein profile was apparent when OMP of $\Delta hutR$ and $\Delta vep20$ were compared with those of the wild-type and the complemented strains (data not shown). As expected, none of the mutations affected the ability to produce siderophores (Table 4).
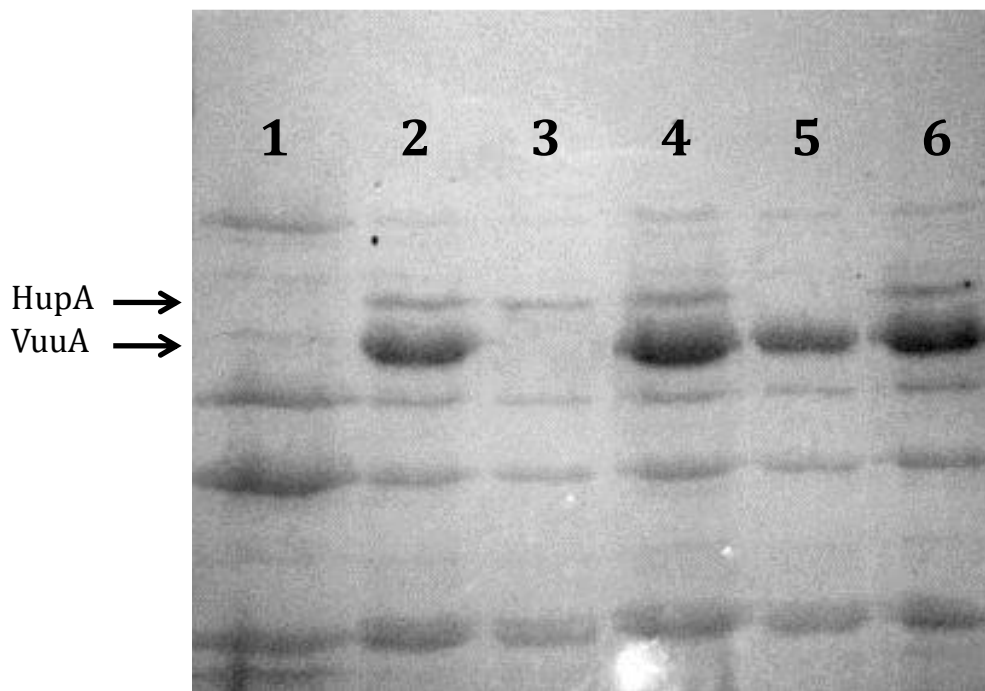
Figure 3. OMP profiles obtained by SDS-PAGE. Lane 1, CECT4999 in CM9-Fe. Lane 2, CECT4999 in CM9-Tf. Lane 3, $\Delta vuuA$ in CM9-Tf. Lane 4, c*vuuA* in CM9-Tf. Lane 5, $\Delta hupA$ in CM9-Tf. Lane 6, c*hupA* in CM9-Tf. Arrows indicate bands of 72 and 77 KDa.

2. Growth in hemin and transferrin

Δ*vuuA* strain was unable to use iron from holo-Tf (Table 4) and was the only strain that did not grow in CM9-Tf after 24 h of incubation, while the other strains grew as well as the wild-type strain. Δ*hupA* and Δ*hutR* strains grew with Hm as the sole iron source but with different growth patterns (Figure 4). Thus, Δ*hupA* strain grew significantly less than the wild-type strain and showed a time-retarded log phase while Δ*hutR* strain grew as efficiently as the wild-type strain (Figure 4). A double mutant in *hupA* and *hutR* was constructed as described in Material and Methods. The double mutant was unable to grow with Hm as the only iron source (Figure 4). In all cases, the complemented strains presented the wild-type phenotype (Table 4 and Figure 4).
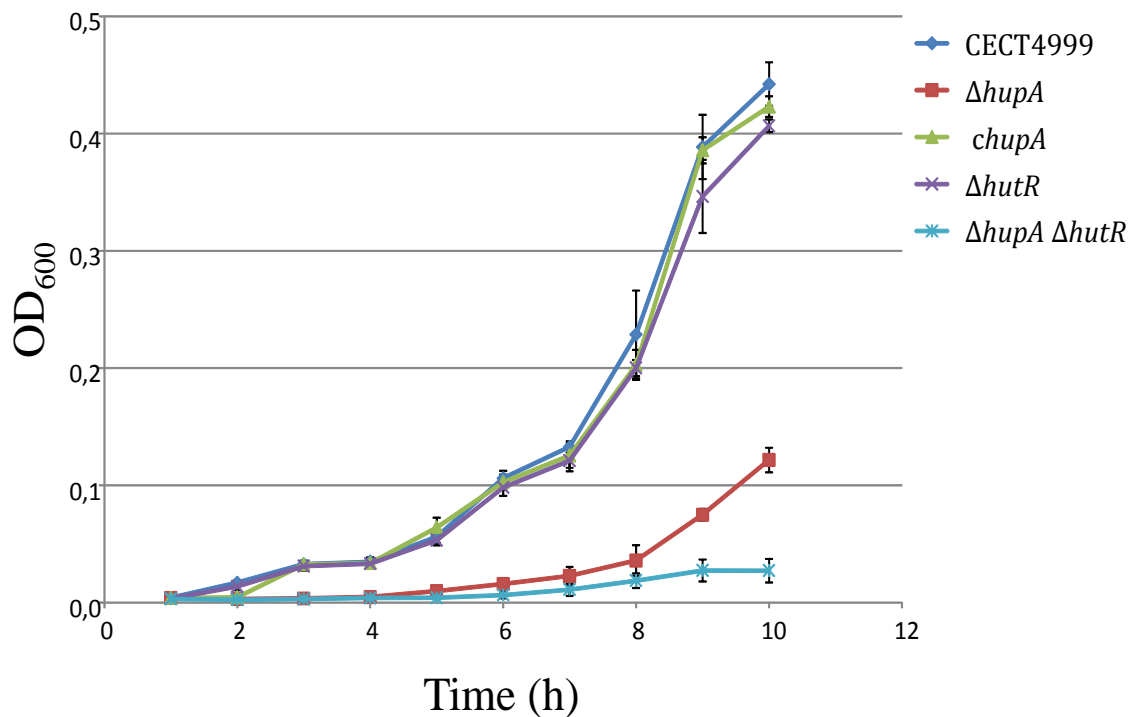
Figure 4. Growth of *V. vulnificus* strains with hemin as sole iron source. Overnight cultures of wild-type strain and its derivative strains were used to inoculate a CM9 supplemented with 100 μM EDDHA and the minimum stimulatory concentration of hemin (0.1 μM). OD_{600} was measured in intervals of 1 hour during 10 hours.

3.   Virulence

The single mutants in *hupA* and *vuuA* showed a similar increase in the LD$_{50}$ values in both animal models (between 1 and 2 log. units) (Table 4). The mutant on *vep20* displayed an increase of only 3-fold higher than wild-type strain in virulence for mice but 4 log units higher in virulence for i.p.-injected eels. Surprisingly, the three mutants were completely avirulent when they were administered to eels through water, which is the natural route of vibriosis transmission (Table 4). In contrast, the single mutant in *hutR* was as virulent as the wild-type strain in both animal models (Table 4) and, in consequence, it was excluded for subsequent experiments.

A double mutant in *hupA* and *vuuA* was found to be completely avirulent for mice and almost avirulent for i.p.-injected eels (Table 4). As expected, the double mutant was avirulent for eels infected through water. Finally, the complemented strains, exhibited the wild-type level of virulence for eels and mice (Table 4).

Table 4. Virulence degree, siderophore production, growth in plasma and with holo-Tf as the sole iron source.

| Strains | Virulence (LD$_{50}$)[1] | | | Siderophore production[2] | | | Growth in fresh plasma from[3] | | Growth with Holo-Tf [4] |
| | Mice | Eels | | Arnow | Csàky | CAS | Humans | Eels | |
		i.p.	bath						
CECT4999	3.16×10^2	2.1×10^2	4.4×10^6	+	-	+	151.3	141.26	17.3 ± 2.8
Δvep20	8.6×10^2	4×10^6	$>10^8$	+	-	+	157.48	0.93	18.3 ± 2.3
ΔvuuA	4.01×10^3	1.0×10^4	$>10^8$	+	-	+	5.68	9.55	0
ΔhupA	8.97×10^3	1.7×10^4	$>10^8$	+	-	+	4.02	3.23	ND
ΔhutR	3.2×10^2	2.0×10^2	5×10^6	+	-	+	ND	ND	ND
cvep20	2.6×10^2	3×10^2	3.6×10^6	+	-	+	114.10	113.07	ND
cvuuA	ND	6.2×10^2	4.1×10^6	+	-	+	180.2	165.5	16.3 ± 2.8
chupA	ND	5.7×10^2	5.6×10^6	+	-	+	178.1	108.6	ND
ΔvuuAΔhupA	$>10^7$	7.4×10^5	$>10^8$	+	-	+	3.73	4.8	ND

[1] LD$_{50}$ for mice was determined by using the iron-overloaded model (Amaro *et al.,* 1994). LD$_{50}$ is expressed as CFU per fish or mouse in case of i.p. injection and CFU per ml in case of bath infection of eels (Amaro *et al.,* 1995).
[2] The criterium for positive or negative result for each test was that of Biosca *et al*. (Biosca *et al.,* 1996).
[3] Ratio between final and initial bacterial counts on TSA-1 plates after 4 hous of incubation in fresh plasma.
[4] Diameter of growth halo in mm around Tf-discs (soaked in a solution of holo-Tf 1mM) placed on CM9A-E plates previously inoculated with 100 µl of an overnight culture in CM9.
ND, non done.

4.   Eel colonization and invasion

To discover whether *vep20*, *vuuA* and/or *hupA* play a role in host colonization and/or invasion (spreading and colonization of the internal organs), the well-established eel model was selected as described in material and methods. In this model, eels are infected by the natural route of disease transmission and the colonization and invasion process is followed by sampling external (gills; the portal of entry into the eel body) and internal (blood, liver, head-kidney and spleen) organs for bacterial counting and quantification of gene expression at different intervals of time post-challenge. The three single mutants were able to colonize the gills and establish a population of similar size to that of the wild-type strain (Figure 5). From this location, the single mutants spread to the internal organs where they survived less than 72 h post-challenge (Figure 5). No significant difference in internal organs colonization degree was detected between Δ*hupA* and Δ*vuuA*, although Δ*hupA* was faster in blood spreading. In contrast, Δ*vep20* presented the lowest invasion capacity since it was not found in blood and spleen at any sampling time (Figure 5). Finally, the double mutant in Δ*hupA* and Δ*vuuA* was able to colonize the gills but failed to spread to internal organs (data not shown).
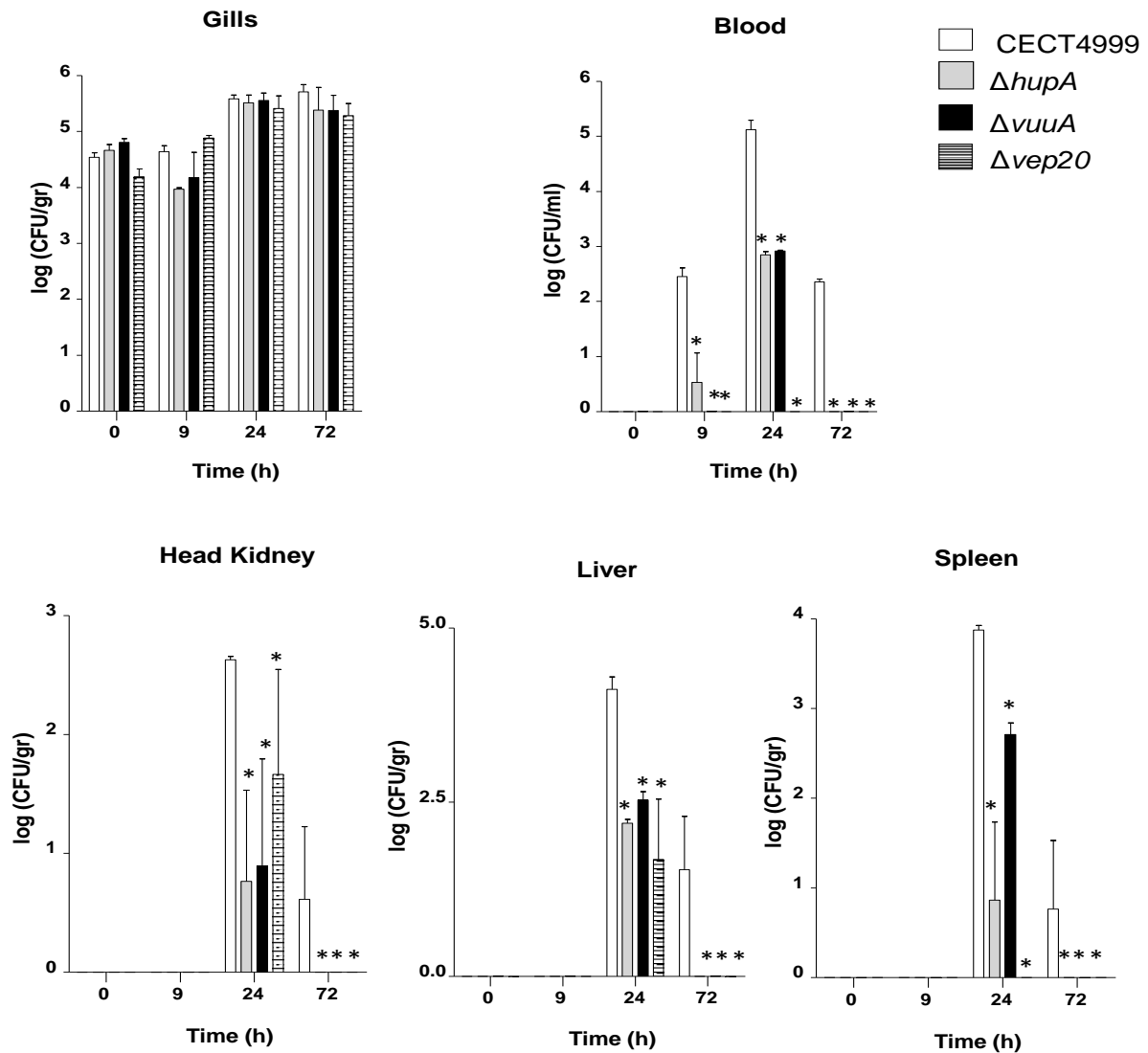
Figure 5. **Eel colonization and invasion assays**. Eels were bath-infected with the wild type strain (CECT4999) or with each one of their single mutants (Δ*vep20*, Δ*hupA* or Δ*vuuA*) at a dose of 10^6 CFU/ml for 1 hour. Then, bacterial colonization degree of external (gills) and internal (blood, liver, head kidney and spleen) organs was measured as bacterial counts (CFU per gr) at 0, 9, 24 and 72 h post-challenge. Asterisks indicate significant differences in bacteria recovered from mutant-strain- and wild-type- strain-infected eels (p<0.05).

In parallel, samples of internal and external organs from eels infected with the wild-type strain were processed to find out whether *vep20*, *vuuA* and *hupA* were overexpressed during the infection process. As observed in Figure 6, overexpression of genes was not induced in gills at any of the assayed times, but was significantly

induced in blood at 9 h and in all the internal organs sampled at 24 h (except *vuuA,* and *vep20* which was not induced in head-kidney), but became undetectable at 72 h post-challenge.
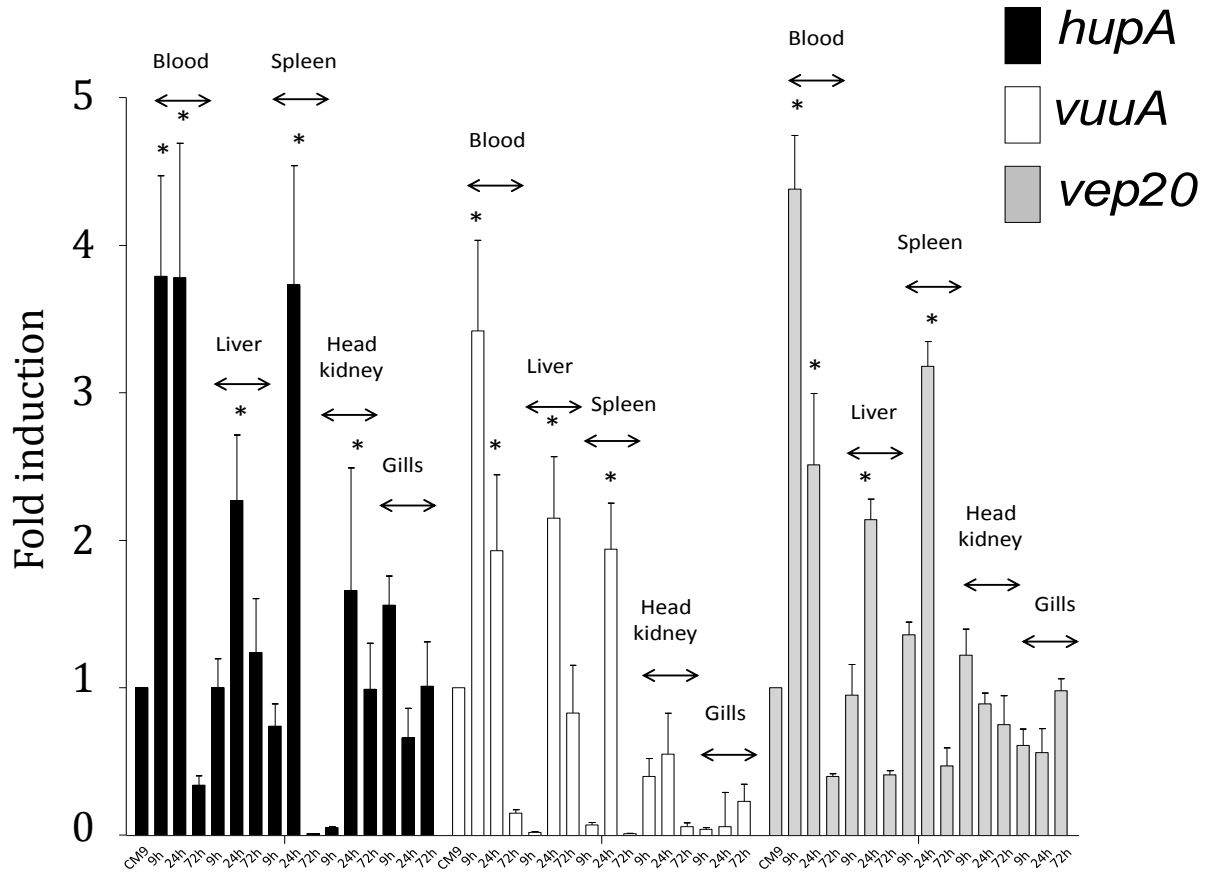
Figure 6. Analysis of gene expression *in vivo* by qRT-PCR. Eels were bath-infected with the wild type strain (CECT4999) at a dose of 10^6 CFU/ml for 1 hour, and gene expression level of *vep20, vuuA* and *hupA* were determined in external (gills) and internal (blood, liver, head kidney and spleen) organs by qRT-PCR at 0, 9, 24 and 72 h post-challenge. Asterisks indicate significant overexpression of each gene with respect to the expression level in CM9 ($p < 0.05$).

5.   Bacterial attachment

The epidemiological data on human and fish infections due to the zoonotic serovar of *V. vulnificus* relate surface wound with severity of infection. To simulate

a superficial wound, eel blood was dried on polyestirene plates and then the attached cells of the wild-type strain and Δ*vep20,* Δ*hupA* and Δ*vuuA* mutants were measured as bacterial DNA by quantitative PCR (qPCR) by measuring the transcriptional level of the housekeeping gene *recA* as reference. No differences in DNA amounts were detected at 0, 3, 9 and 24 h (data not shown), suggesting that the lack of these genes did not affect the cell attachment on wounds in the assayed conditions.

6.   Growth in plasma and blood

Single and double mutants in *hupA* and *vuuA* were able to survive and grow in fresh eel and human plasma, although at significantly lower rates than the wild-type and the complemented strains (Table 4 and Figure 7). Eel plasma was the condition selected to demonstrate that the reduction in growth rate was due to the bacteriostatic effect of transferrin and not to the bacteriolytic action of complement. As expected, significant differences in bacterial growth between each mutant and the wild-type strain were still found after complement inactivation but not after iron supplementation (Figure 7). Finally, the complemented strains showed a similar growth rate to the wild-type strain in all the assayed conditions (data not shown). With regard to Δ*vep20,* the mutant did not grow in eel plasma but did present a wild-type growth in human plasma.
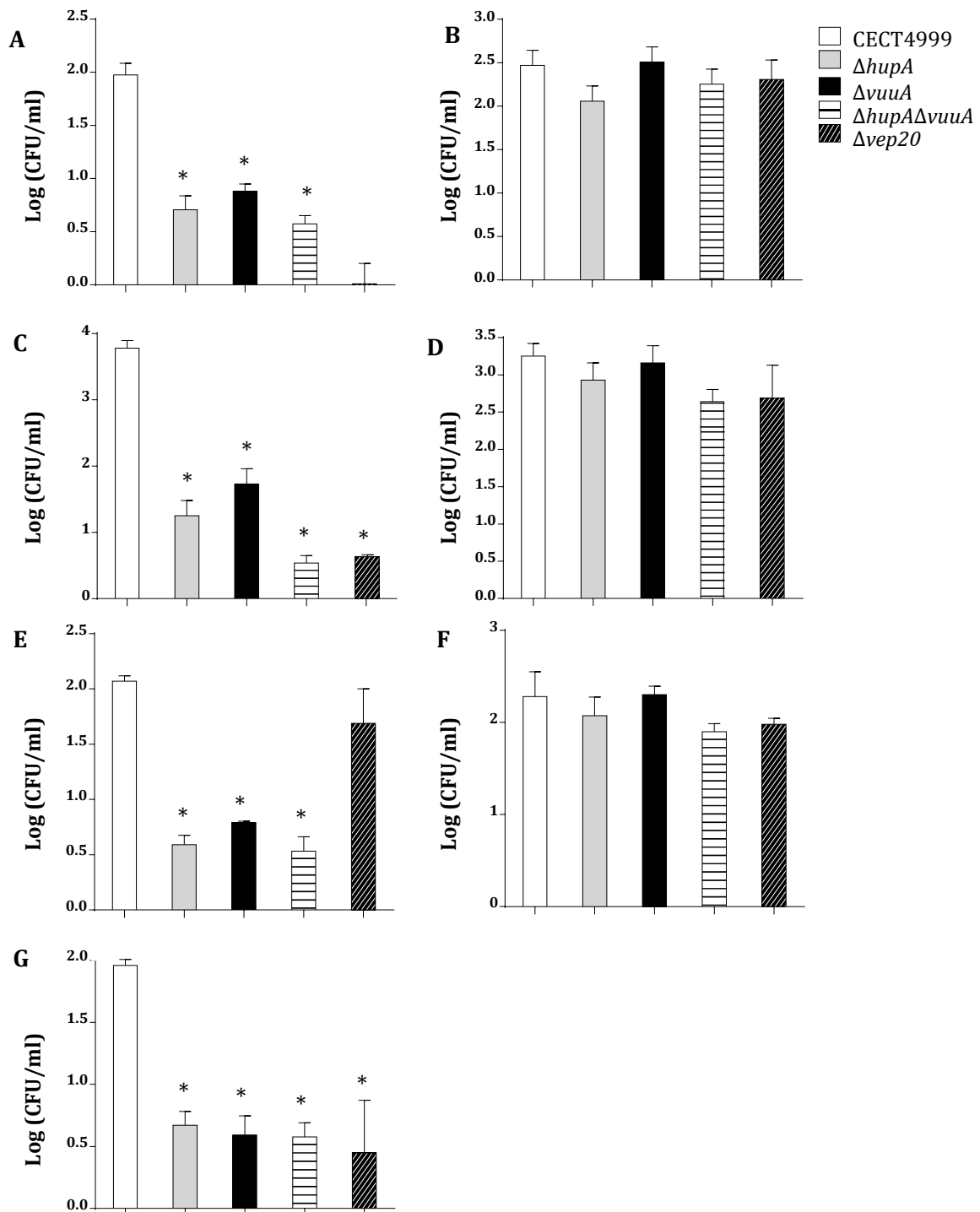
Figure 7. Growth in plasma and blood. Bacterial growth of *V. vulnificus* strains presented as increase of CFU/ml, expressed as $\log_{10}$ units, after 4 hours of incubation. A) fresh eel plasma; B) fresh eel plasma + $FeCl_3$; C) inactivated eel plasma; D) inactivated eel plasma + $FeCl_3$; E) fresh human plasma; F) fresh human plasma + $FeCl_3$ and G) eel blood. Asteriks indicate significant differences in growth between the mutant and the wild-type strain ($p<0.05$).

7. Phagocytosis and destruction of phagocytes and erythrocytes

To discover if Vep20 has some role in resistance to phagocytosis, a phagocytosis assay was performed with the wild-type strain and its mutant. The bacterial survival of both strains both inside and outside the PEC was similar at all sampled times and was independent of previous opsonization (Figure 8A and B). Thus, both strains were not observed inside phagocytes unless they were previously opsonized, and, in this case, without significant differences in internalized bacterial numbers (Figure 8C).
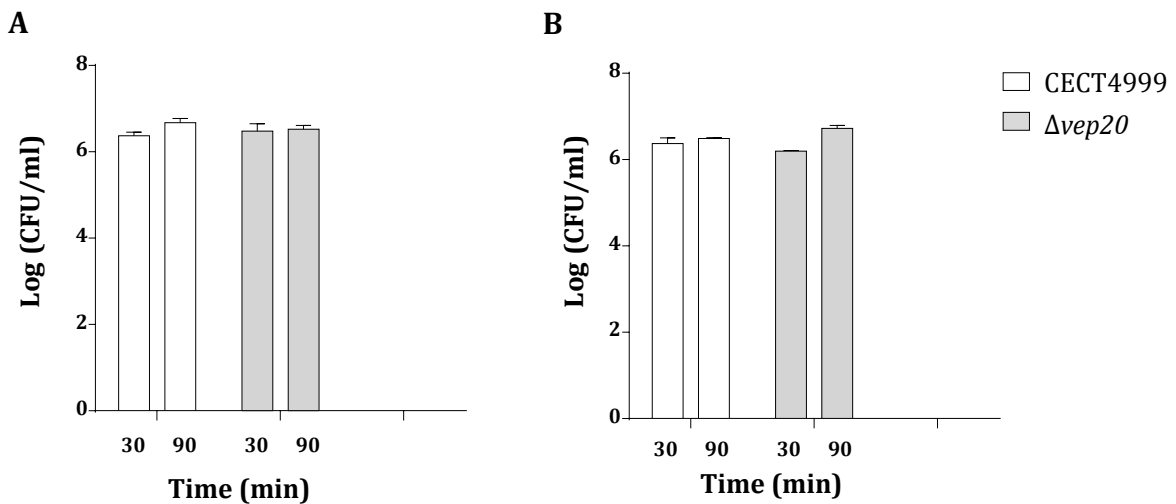
Figure 8. Phagocytosis assay. Number of total (extra- and intracellular) bacteria after 30 and 90 min of incubation of CECT4999 and Δ*vep20* strains in monolayers of fresh eel PEC at a moi of 10, where bacteria where opsonized (B) or not (A). (C) Number of intracellular opsonized bacteria, incubated with PEC as described for A and B.
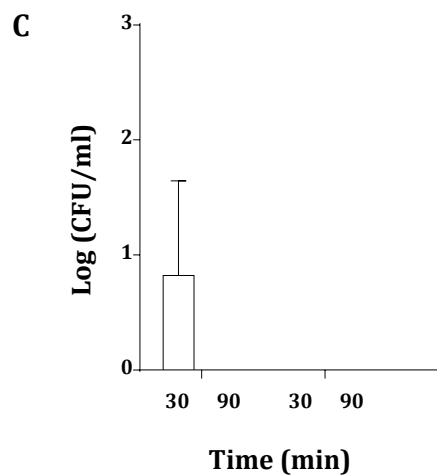
Finally, microscopic observations of PEC incubated with wild-type and Δ*vep20* mutant strain also showed that both strains were able to lyse all the cells in less than 90 min (Figure 9A), and that no bacteria were located inside the phagocytes stained with Hemacolor in all tested strains (Figure 9B).
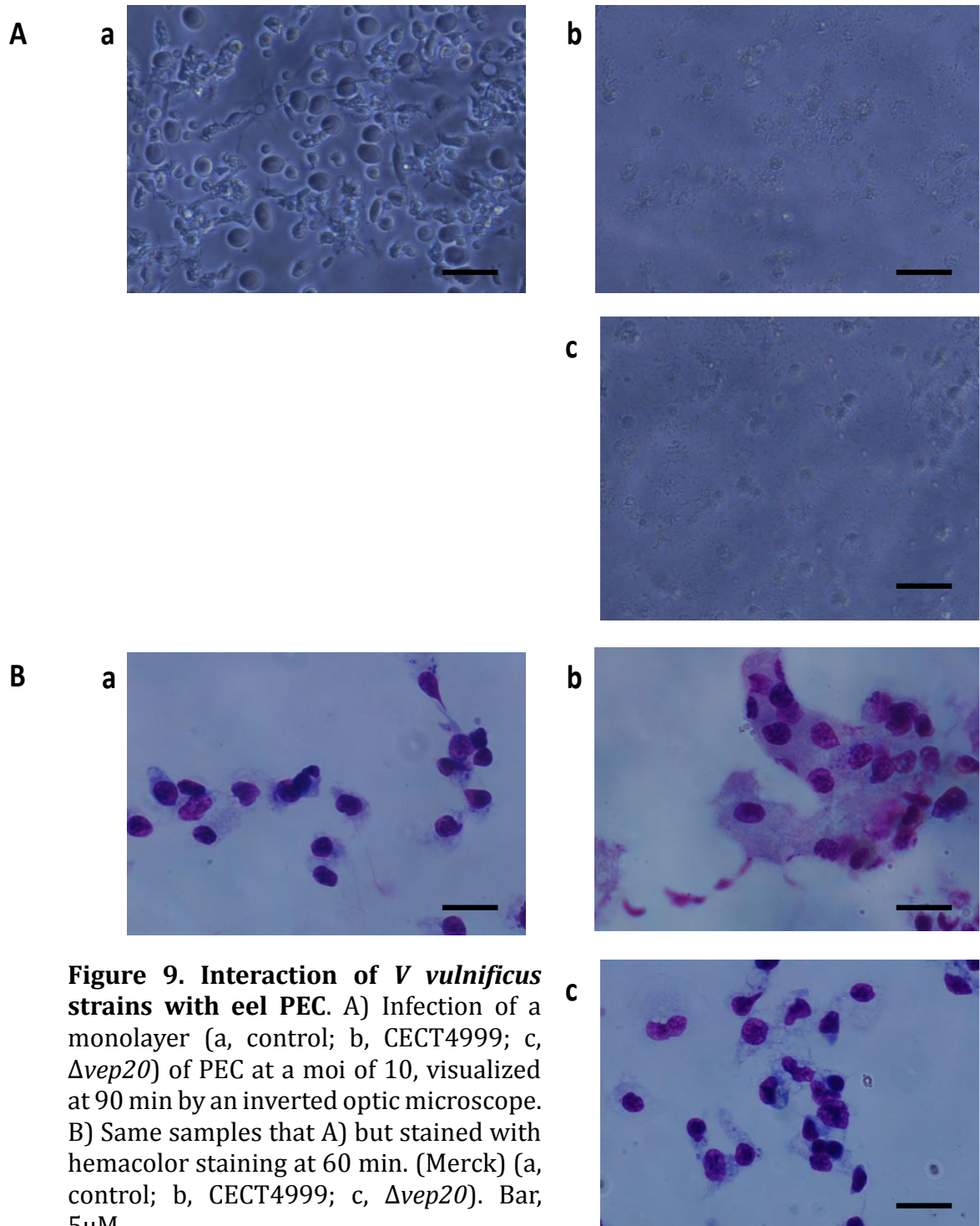
Figure 9. Interaction of *V vulnificus* strains with eel PEC. A) Infection of a monolayer (a, control; b, CECT4999; c, Δ*vep20*) of PEC at a moi of 10, visualized at 90 min by an inverted optic microscope. B) Same samples that A) but stained with hemacolor staining at 60 min. (Merck) (a, control; b, CECT4999; c, Δ*vep20*). Bar, 5μM.

In parallel, the wild type strain and Δ*vep20* mutant were incubated in presence of eel erythrocytes. Direct observation of the interaction of bacteria with eel erythrocytes were made at 90 min post-infection. At this time both the wild-type and Δ*vep20* mutant strains destroyed all the erythrocytes without significant differences between them (Figure 10).
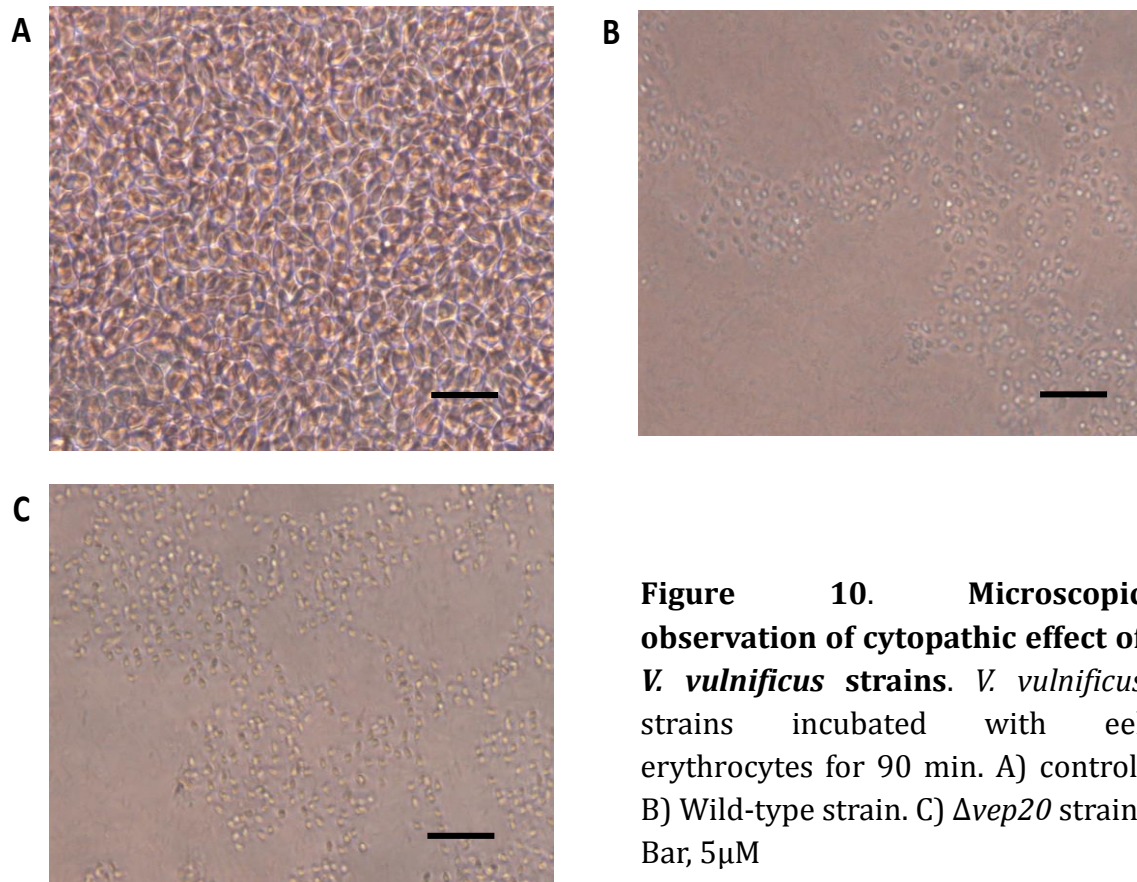
Figure 10. Microscopic observation of cytopathic effect of *V. vulnificus* strains. *V. vulnificus* strains incubated with eel erythrocytes for 90 min. A) control. B) Wild-type strain. C) Δ*vep20* strain. Bar, 5μM

C. Phylogeny of *vep20*, *hupA* and *vuuA*

The genes *vep20*, *vuuA* and *hupA* were sequenced in a collection of *V. vulnificus* strains from clinical and environmental sources belonging to the three biotypes and the three previously defined phylogroups (Sanjuán *et al.,* 2011). The gene *vep20* was identical in all the strains (all of them of biotype 2), which demonstrates that this gene has not evolved. The variability was higher for *vuuA* and *hupA*. Thus, the phylogenetic reconstruction using the maximum-likelihood (ML)

method showed that the *vuuA* gene has two main variants (Figure 11): *vuuA(I)* is present in 26 of the 29 studied strains, including the sequenced strain YJ016 (of Bt1 and clinical origin) and all the Bt2 and Bt3 strains; *vuuA(II)* is present in a few environmental and clinical Bt1 strains, including the sequenced strain CMCP6 (Kim *et al.,* 2003). The percentage of inter-variant identity in both DNA and protein sequence is around 80-85% while the intra-variant identity is between 89.7 and 90.3% for *vuuA(I)* and between 97.7 and 98.1% for *vuuA(II)*.
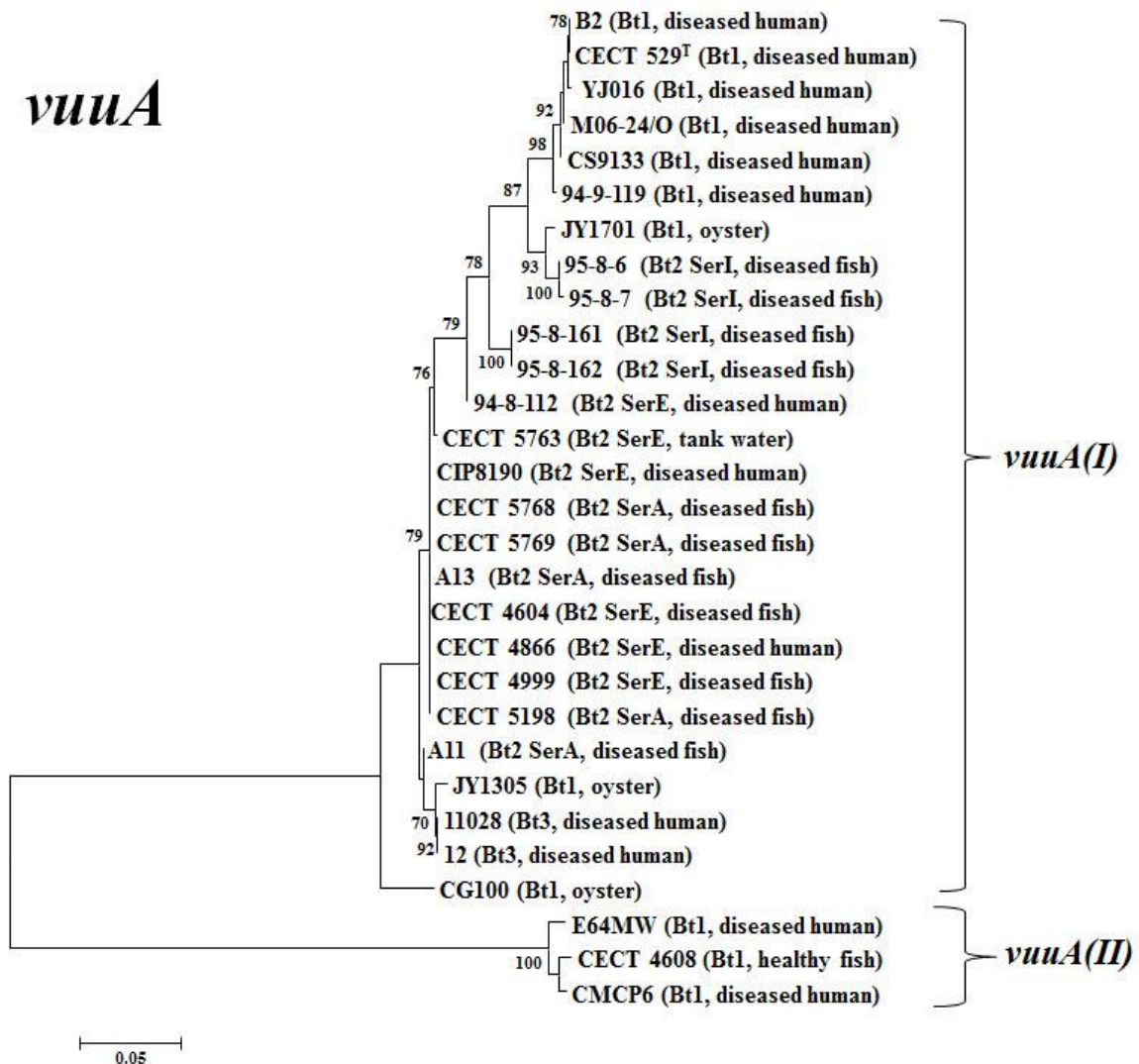
Figure 11. Phylogenetic tree of *vuuA*. Maximum-likelihood tree derived from the *vuuA* gene. Bootstrap support values higher than 70% are indicated in the corresponding nodes.

The gene *hupA* also presents two main variants (Figure 12): *hupA(I)* was found in all strains from diseased fish and clinical cases associated to fish manipulation; *hupA(II)* also has two subforms, one defective because it lacks a fragment of 2035 nt in the 5′ portion of the gene [*hupA(IIa)*], and the other complete [*hupA(IIb)*] (Figure 12). The percentage of inter-variant identity in both DNA and protein sequence was between 91.6 and 95.6% while the intra-variant identity was from 96.1 to 95.9% for *hupA(I)* and 95 to 95.1% for *hupA(II),* being 100% for *hupA(IIa)* and between 95 and 95.1% for *hupA(IIb).*
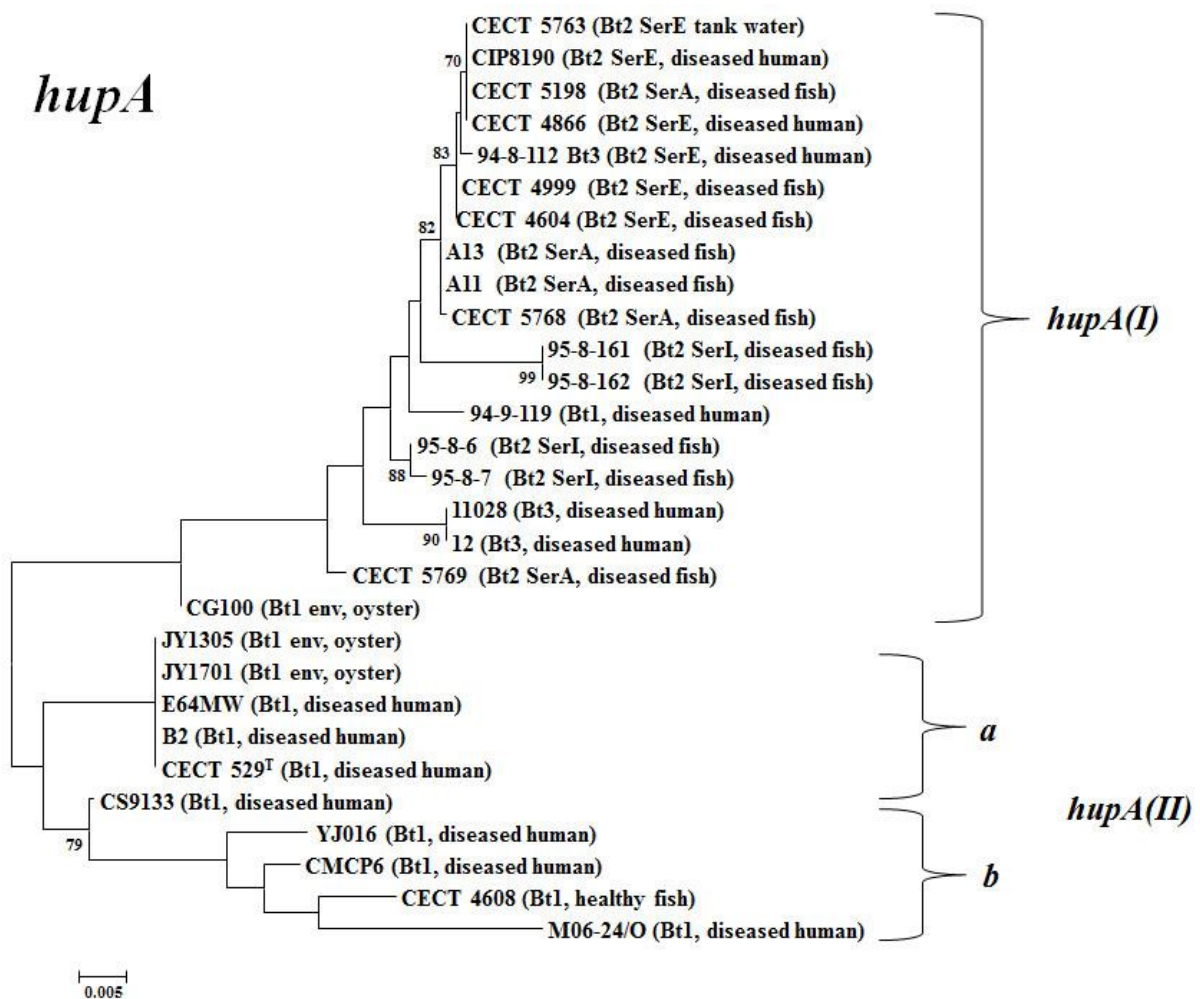
Figure 12. Phylogenetic tree of *hupA*. Maximum-likelihood tree derived from the *hupA* gene. Bootstrap support values higher than 70% are indicated in the corresponding nodes.

The sequences of both genes were compared to identify the regions where the mutations accumulated. As shown in Appendix I and II, variations were detected throughout the protein. Meanwhile *vuuA* presented changes in 156 amino acids (63.5% amino acids of different families; 36.5% amino acids of the same family) while *hupA* showed variations in 41 amino acids (68.3% amino acids of different families and 31.7% amino acids of the same family).

The phylogenetic trees for each gene were compared with that obtained by MLSA from the selected housekeeping and virulence-related genes to discover whether their phylogenetic histories were congruent with one another. Figure 13 shows the MLSA tree, which divides the population into two main clades, unrelated with biotype or origin of the isolate (Figure 13). The results of the Shimodaira-Hasegawa (SH) and expected-likelihood weight (ELW) tests are summarized in Table 5. All the comparisons were highly significant for both tests, which would indicate that the phylogenetic reconstructions obtained from each gene are congruent with one another and with the MLSA, thus providing statistical support for similar evolutionary rates.
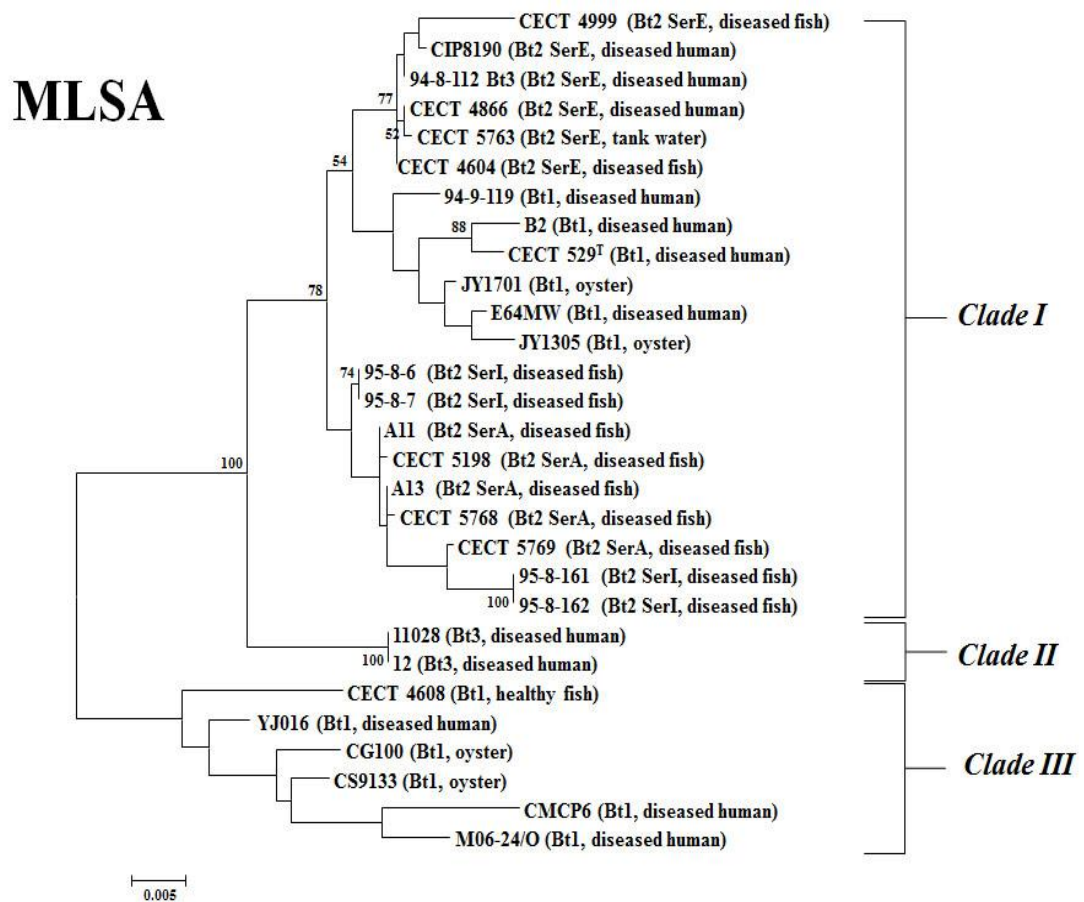
Figure 13. Phylogenetic tree of MLSA. MLSA tree using the T92+GI mode of evolution. Bootstrap support values higher than 70% are indicated in the corresponding nodes.

Table 5. Summary of Shimodaira-Hasegawa (SH) and expected likelihood weights (ELW) for the MLSA sequences and *vuuA* and *hupA* genes.

Alignment	Topology	lnL[a]	SH test[b]	ELW test[b]
MLSA	MLSA	-7732.85	1.000	1.000
	vuuA	-6246.04	1.000	0.0056
	hupA	-7757.89	1.000	0.9589
vuuA	*vuuA*	-4970.60	1.000	0.6663
	MLSA	-4347.71	1.000	0.1373
	hupA	-4539.55	1.000	0.4489
hupA	*hupA*	-4553.87	1.000	0.0289
	vuuA	-4842.49	1.000	1.000
	MLSA	-4717.26	1.000	0.9205

We also estimated the time of divergence by using sSNP (Akaike, 1974). The number of sSNP for the *hupA* gene ranged from 0 to 86, with an average of 22 sSNP/strain. In the case of *vuuA* type I, the average number of sSNP was 49/strain (ranging 0 to 119) and for type II was 21/strain (ranging 16 to 26). The potential sSNP sites were 2047 for *hupA*, 2018 and 1962 for *vuuA* types I and II, respectively. These numbers were used to calculate the molecular clock, the results of which are shown in Table 6. According to the model used, based on *E. coli* (365 generations per year and a mutation rate of 5.4×10^{-10}), Bt1 strains diverged from each other an average of 55,000 years ago, whereas strains within the other groups diverged from 0 (Bt3) to 13,479 (Bt2) years ago. For *vuuA* type I, Bt1 strains diverged from each other an average of 57,000 years ago, whereas strains within the other groups diverged between 0 (Bt3) to 42,000 (Bt2) years ago, and for type II from 20,000 to 32,000 (Bt1 strains) years ago.

Table 6. Average time of divergence (years) for the *hupA* and *vuuA* genes based on sSNP analysis taking 365 generations per year and a mutation rate of 5.4×10^{-10}. Bt, biotype.

hupA					
	Bt1	Bt2serE	Bt2serI	Bt2serA	Bt3
Bt1	55295.0	63173.2	61476.6	61786.4	74710.2
Bt2serE	63173.2	1321.9	19828.3	6237.7	29329.4
Bt2serI	61476.6	19828.3	20447.9	19518.5	29432.6
Bt2serA	61786.4	6237.7	19518.5	8303.1	26768.2
Bt3	74710.2	29329.4	29432.6	26768.2	0.0

vuuA type I					
	Bt1	serE	serI	serA	Bt3
Bt1	56847.9	85458.0	65612.5	89392.2	931634
Bt2serE	85458.0	13744.1	73067.7	9134.8	31007.9
Bt2serI	65612.5	73067.7	42950.2	79195.9	7416.6
Bt2serA	89392.2	9134.8	79195.9	4022.6	21873.2
Bt3	93163.4	31007.9	74167.6	21873.2	0.0

vuuA type II (all strains are of biotype 1)				
		CMCP6	CECT4608	E64
	CMCP6	0	20113.2	27655.7
	CECT4608	20113.2	0	32684.0
	E64	27655.7	32684.0	0

D. Analysis of Vep20 protein

The analysis of the aminoacid sequence of Vep20 by various programs revealed relevant information. This gene was annotated as a putative Tf-receptor on the basis of its low similarity with a gene that encodes a putative Tf-receptor in *Histophilus somni*, one of the key bacterial pathogens involved in the multifactorial etiology of the Bovine Respiratory Disease Complex (Corbeila, 2008). The last BlastP search revealed that the highest homology for this protein is showed by a series of putative Tf/Hb-binding proteins of different human and fish pathogens (*V. harveyi*,

Photobacterium damselae, Neisseria meningitidis and *Bordetella* sp.). Pfam predicted that Vep20 contains 741 aminoacids and presents two conserved functional domains; the TonB-dependent receptor domain, a beta-barrel structure that forms a channel across outer membrane by which the ligand enters into the cell and the plug domain, which putatively acts as the gate allowing or preventing the entry of the ligand through the and the beta-barrel. Regarding to the cellular location, PsortB and Secretome revealed that Vep20 sequence matched to an outer membrane protein possessing a signal peptide needed to be inserted into the outer membrane.

The protein Vep20, a protein of about 79 KDa, was detected by immunostaining after OMP separation by electrophoresis and transference to PVDF membrane (Figure 14A). This molecular weight corresponds to that predicted for Vep20 by the *in silico* analysis taking into account that proteins are modified after translation and show a molecular weight slightly lower than predicted *in silico,* in this case 84 KDa. As Figure 14 shows, Vep20 was induced in artificial medium under the iron restricted conditions imposed by both 40 μM of purified human apo-Tf, and, as expected, the addition of iron to the medium abolished the production of Vep20 (Figure 14B). Vep20 was detected in the membrane extractions, total and outer (Figure 14C), and eel plasma added to the medium at a minimum of 40% (vol/vol) also induced the expression at translation level (Figure 14D). Finally, Vep20 was detected in CM9-EP from 4 h of incubation (Figure 14E). The complemented strain gave the phenotype of the wild-type strain in all the assays.
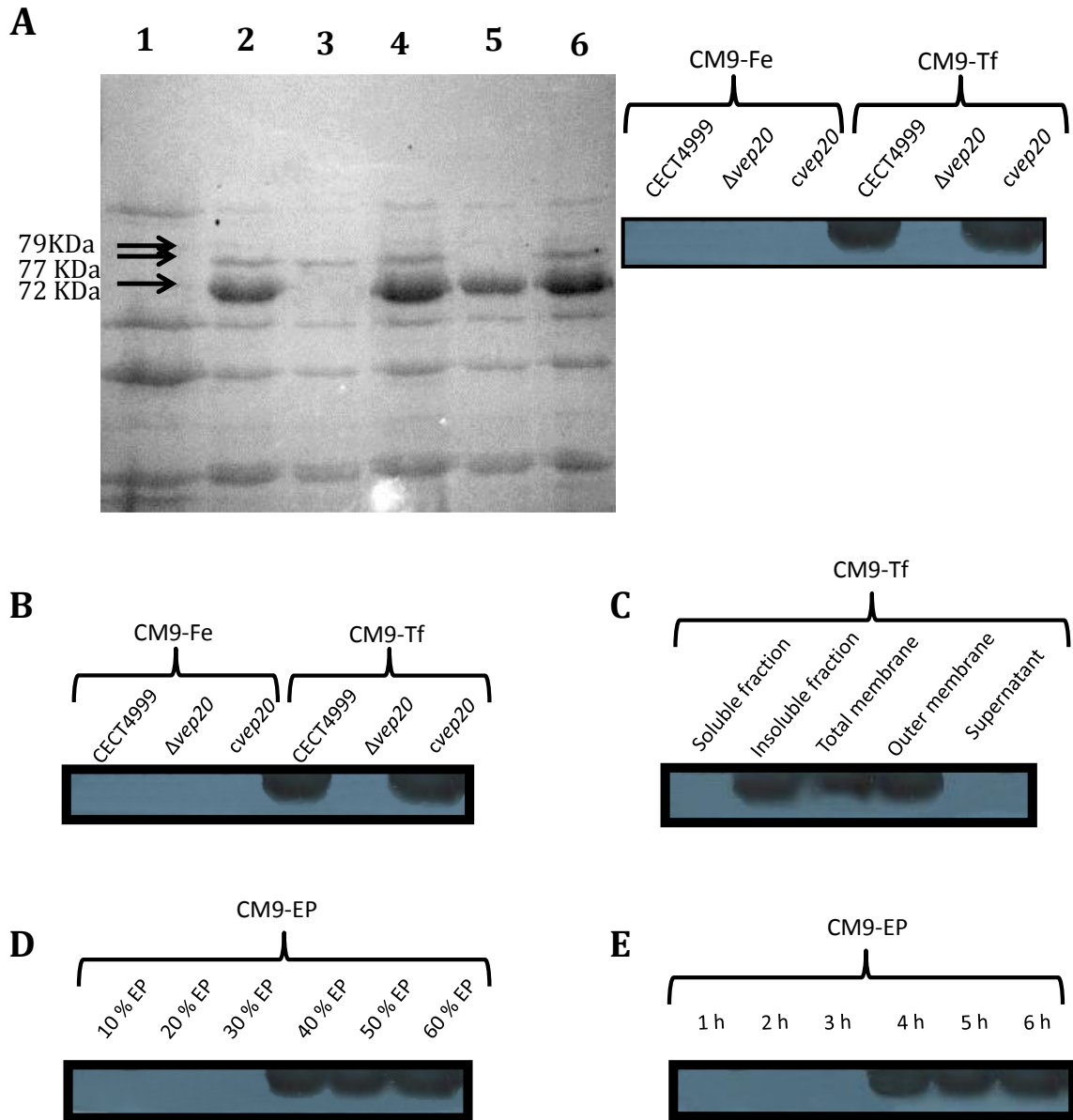
Figure 14. **Western blot analysis using polyclonal antibodies specific to Vep20 protein**. A) Comparison between an iron-regulated OMP comassie-stained SDS PAGE (same that Figure 3) with a western blot of Vep20 protein revealed with specific antibodies. B) Expression in presence/absence of iron. C) Cellular location of Vep20 protein. D) Expression of Vep20 protein in CM9 using several percentage of eel plasma. E) Time course expression analysis of Vep20 protein in CM9-EP (CM9 plus 50% EP).

IV.   DISCUSSION

The present work focused on the iron-acquisition systems that the zoonotic serovar of *V. vulnificus* employs to infect both humans and fish. To do so, mice and eels were chosen as animal models to test the role these mechanisms play in virulence. Previous studies on the iron-uptake mechanisms of *V. vulnificus* Bt2, and in particular the zoonotic variant, suggest that it is able to produce phenolate and hydroxamate-type siderophores and use Hm as sole iron source (Biosca *et al.,* 1996; Fouz *et al.,* 1996). On the basis of siderophore production by Bt1, it was hypothesized that Bt2 strains produce vulnibactin and a new hydroxamate-type siderophore (Biosca *et al.,* 1996). The genes for biosynthesis and uptake of vulnibactin were identified by FURTA but no gene related to hydroxamate production was detected. This finding was further confirmed by performing specific tests for siderophore detection, which were only positive for phenolate production. Thus, the selected strain of the zoonotic serovar only produces vulnibactin, demonstrating that there are differences in siderophore production among strains of the same clonal complex. Additional identified genes were those related to exogenous aerobactin uptake, previously identified in the Bt1 of the species (Tanabe *et al.,* 2005), as well as those related to Hm uptake, which would constitute the genetic basis for this previously reported ability (Fouz *et al.,* 1996).

The hypothesis of the present study was that the iron-uptake systems from vulnibactin and Hm are host-nonspecific virulence factors. The selected genes (*vuuA*, *hupA* and *hutR*) were sequenced and the corresponding proteins showed a similarity value of more than 95% with regard to the clinical Bt1 strain used as reference. The single mutants and corresponding complemented strains were obtained by allelic exchange and were phenotypically evaluated in terms of siderophore production, OMP profiles and growth in the presence of holo-Tf or Hm as sole carbon sources. In general terms, the phenotype obtained was the expected one. Thus, vulnibactin production was not affected by any of the three mutations, the OMP profiles from Δ*vuuA* and Δ*hupA* lacked the corresponding predicted band and Δ*vuuA* was unable to grow in the presence of holo-Tf as sole iron source. With respect to *hutR*, we did not detect differences in OMP profiles between the mutant

and the wild-type strain, which correlates with the results obtained by Datta and Crosa (Datta and Crosa, 2012), who suggest that HutR is a minority protein in the OM. Regarding iron-uptake from Hm, we found that *hupA* is the gene mainly involved in this system, since its disruption significantly diminished the ability to grow with Hm as sole iron source. However, *hutR* is also needed to completely abolish growth ability *in vitro.* This result is also compatible with those obtained by Datta and Crosa (Datta and Crosa, 2012) who suggest that *hutR* plays a secondary role in the use of Hm by *V. vulnificus* Bt1. In parallel, we confirmed that the three genes were overexpressed under iron-restriction conditions and that *hupA* was significantly more expressed than *hutR*, a result again in concordance with the hypothesis that *hutR* is secondary in Hm uptake. The finding that *vuuA* and *hupA* were maximally induced in the log. phase of growth would suggest that they are probably involved in active growth, both *in vivo* and *in vitro.* Finally, the complemented strains showed the phenotype of the wild-type strain, demonstrating that in each case the mutation only affected the target gene(s).

The results obtained in the virulence assays support the hypothesis on the role of *hupA* and *vuuA* as host-nonspecific virulence genes. Thus, in single-gene (*hupA* or *vuuA*) knockout mutants, virulence was attenuated by 1-2 logs for both i.p.-injected eels and mice, while virulence was completely abolished for eels when bacteria were administered by water, the natural route for vibriosis transmission. By contrast, *hutR* was found not to be a virulence gene since its mutation did not affect the lethal dose for either animal model. This result is compatible with those obtained *in vitro* and also supports the hypothesis posed by Datta and Crosa (Datta and Crosa, 2012). Interestingly, the double-gene (*hupA* and *vuuA*) knockout mutant was completely avirulent for mice and almost avirulent for eels, both inoculated by the i.p. route and bath immersion, suggesting that iron-acquisition by either ferric vulnibactin or heme uptake is absolutely needed for the zoonotic serovar to cause septicemia in mice. Regarding eels, the remaining virulence of the double mutant could be due to a third iron-acquisition system, in this case, host-specific.

On the basis that *vuuA* and *hupA* are virulence genes, the next step was to discover their specific roles in human and fish vibriosis. To this end, we performed a series of *in vivo* and *in vitro* experiments under the hypothesis that this pathogen

needs both genes to grow in host blood and internal organs and achieve the population size that triggers host death by sepsis. Firstly, both genes were overexpressed in eels after bath infection, which demonstrates that both are required *in vivo*. This overexpression was only detected in internal organs (blood, spleen and liver) and from 9 (blood) to 24 h (blood, spleen and liver) post-infection, which suggests that VuuA and HupA are used *in vivo* during the first 24 h of infection. After this time, cellular destruction caused by the pathogen would release iron from cellular storage depots that could be used for bacterial growth (Valiente *et al.,* 2008c). This result is also compatible with the hypothesis of Lee and cols. (Lee *et al.*, 2012), which suggests the bacterium needs a minimum of 24 h to spread from gills to the internal organs and achieve the population size that triggers death by sepsis. Then, we analyzed the effect of single mutations in *vuuA* or *hupA* and that of the double mutation in both genes on surface and internal colonization of eels. We found that all the mutant strains, single and double, were able to colonize the gills as efficiently as the wild-type strain. However, each of the single mutants was deficient in internal colonization. In fact, the single mutants grew significantly less than the wild-type strain in each organ and were completely eliminated from internal organs at 72 h post-infection. This result explains why they were not virulent by bath challenge and suggests that a minimal bacterial growth inside the body is needed by *V. vulnificus* to overcome the immune defenses. In addition, the double mutant strain completely lost the ability to spread from the gills to the internal organs, confirming the importance of iron-acquisition by either system for colonization and invasion. Likewise, either one or the other gene was needed for efficient growth in human and eel blood and plasma. In fact, both genes were overexpressed by the wild-type strain in fresh plasma from both humans and eels, which correlates with the results obtained *in vivo* and supports the hypothesis on the role played by both iron-uptake systems in the ability of this zoonotic serovar to grow in blood and cause death by sepsis.

According to the virulence results, the double mutant in *vuuA* and *hupA* was completely avirulent for mice but retained some virulence degree for eels. This question was quite intriguing and lead us to investigate if there was another iron-uptake mechanism, in this case specific for fish. *V. vulnificus* Bt2 possesses a

virulence plasmid that encodes resistance to the eel innate immunity by unknown mechanisms. In fact, the genes that could be involved in this resistance encode putative proteins without similarity or with low similarity to known proteins. One of this ORF, *vep20*, is the only one that shows significant homology to a series of proteins with a putative role in resistance to nutritional immunity: Tf/Hb-binding proteins. To discover if this protein could be a receptor specific for eel Tf, we performed a series of additional experiments. First, we obtained the single mutant and tested it for virulence and eel colonization and invasion. Interestingly, the virulence for mice was not affected while the virulence for eels was significant diminished in 4 log. units. In parallel, the growth ability of the mutant in HP was not altered while the EP exerted a bacteriostatic effect on the mutant, which could be attributed to a deficient iron-uptake from eel transferrin. In accordance, the mutant was able to colonize the gills but was unable to grow in eel blood. The protein was *in silico* analyzed and its predicted structure corresponds to a membrane protein with two domains, one β-barrel and the other a plug-domain, compatible with a Tf-receptor. We obtained the recombinant protein and immunized mice to get specific antibodies. The rabbit antibodies identified a protein of around 78-80 KDa in the outer membrane fraction. Then, the transcription and translation of *vep20* was analyzed. The gene was induced in iron-restriction and in presence of EP and was maximally induced in the log. phase of growth. In addition, Vep20 was detected from 4 h of growth in iron-restriction conditions. Additional experiments of resistance to the innate immunity such as resistance to phagocytosis and destruction of erythrocytes and phagocytes confirm that Vep20 is only involved in growth in iron-restriction. In conclusion, all these findings support the hypothesis that Vep20 is a receptor specific for eel-Tf that collaborates in the resistance to the innate immunity conferred by the plasmid. Further experiments of specific binding to recombinant eel-Tf are needed to confirm this attractive hypothesis.

Our next step was to analyze the phylogeny of *vuuA* and *hupA* and compare it with that of the species to discover whether both genes are part of the accessory genetic elements, as *vep20*, or part of the core genes. Interestingly, the phylogenetic trees for each gene were congruent with each other and with the species constructed from the four housekeeping and four virulence-related genes. This

result strongly suggests that *vuuA* and *hupA* are part of the core genes of the species and have not been acquired through horizontal gene transfer, as occurs with siderophore- and heme-related iron-uptake in other bacterial pathogens (Martínez, 2013). The divergence time for each one of the genes, calculated by the SNP analysis, was also congruent with the evolutionary scenarios provided by phylogenetic trees. In all cases, Bt1 was found to be the more heterogeneous group, showing the highest theoretical divergence time. The evolutionary scenario also shows that Bt1 was the first to emerge, followed by Bt2-SerE, -SerA, -SerI and, finally, Bt3. Interestingly, *vep20* was identical in all the biotype 2 strains analyzed irrespective of its clonal origin. This finding supports the previous hypothesis about the polyphyletic origin of this biotype by acquisition of the virulence plasmid by different *V. vulnificus* clones in the fish farming environment. In addition, the fact that *vep20* does not show any variation in sequence also supports that the plasmid has been acquired very recently and/or the gene *vep20* is under a strong selective pressure that precludes any change in the nucleotide sequence. This last hypothesis underlies the important role of *vep20* in host-specific virulence and supports a scenario in which the host adaptation in *Vibrio* is driven by HGT phenomena produced in its natural ecosystem.

The fact that *vuuA* and *hupA* belong to the core of the species also suggest that they probably play a role not only in virulence but also a general in survival outside the hosts of vibriosis. Accordingly, we found two main polymorphic variants for both genes without an apparent relationship with biotype or origin (clinical versus environmental) of the isolate. However, a deeper study of the origin of the isolates provided evidence of some kind of relationship between receptor variant and environment. Thus, for *hupA,* all the strains that produced *hupA(I)* came from fish farming-related environments (diseased fish, tank water, healthy fish and humans infected through fish handling), which would suggest that *hupA* could have diverged as a consequence of better adaptation to Hm-containing fish proteins. On the other hand, in *vuuA*, this adaption to the environment was mainly evident for the zoonotic strains. In this case, the theoretical divergence time for the gene was much longer than that expected for a clonal complex. The most plausible explanation would be that the environment acts as a strong selective force because the main source of

variation for this clonal complex is the multiplicity of environments from which the strains were isolated (water, healthy fish, diseased fish, human expectoration, human wound, human septicemia...). The adaptation to the environment of a siderophore receptor could be a consequence of changes in the siderophores, produced by mutations in the biosynthetic genes, due to the competence by iron in the natural environments of the bacteria. The same hypothesis was proposed to explain the variation in receptors for pyoverdin in *Pseudomonas* spp. (Bodilis *et al.,* 2009). Another interesting observation provided by the phylogenetic study was that some Bt1 strains from clinical and environmental sources presented a truncated form of the *hupA* gene. Interestingly, these strains possess a whole *hutR* gene, which suggests that they could use this second receptor to uptake iron from heme-proteins. This finding provides a biological explanation for the presence of a second gene for heme-receptor in the genome of the species.

In conclusion, *vuuA* and *hupA* are host-nonspecific virulence genes involved in the colonization and invasion of internal organs by enabling the bacterium to grow in the iron-restriction conditions imposed by the mammal and teleost hosts, while *vep20* is a fish-specific gene involved in colonization and invasion of the eels by probably binding eel-Tf. This work also demonstrates that iron-uptake from hemin and/or vulnibactin is essential to cause vibriosis in mice and suggests that probably a third host-specific system could also be involved in sepsis in fish. The phylogenetic study also suggests that *vuuA* and *hupA* genes are part of the core genes of the *V. vulnificus* species and are subjected to variations, probably due to environmental adaptations while *vep20* was acquired by HGT conferring, together with the rest of plasmid genes, an adaptation to a new host; the eel. This gene has not varied since its acquisition, which suggests that this has been very recent and also that the genes is under a strong selective force probably exerted by the host. Finally, *hutR* encodes a secondary heme receptor that is not relevant to virulence, although it could be used by the strains with a truncated form of *hupA*, like those we have found in this study.

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CHAPTER 3

MICROARRAY ANALYSIS OF THE ZOONOTIC SEROVAR OF *Vibrio vulnificus*

I. INTRODUCTION

In the previous chapter, we found evidences that iron could be one of the common signals that *V. vulnificus* "senses" in mammal and fish tissues. The main regulator that responds to external iron levels in bacteria is Fur (Iron uptake regulator). Recent studies indicate that Fur, in addition to regulate iron-uptake, controls multiple genes involved in a variety of cellular processes such as acid shock response, chemotaxis, metabolic pathways, bioluminescence, and the production of toxins and other virulence factors (Kim *et al.,* 2005; Kim *et al.,* 2013a; Septer *et al.,* 2013; Carpenter *et al.,* 2009). In some bacteria, Fur can also act as a positive regulator in controlling gene expression (Carpenter *et al.,* 2009).

The regulator Fur of *V. vulnificus* biotype 1 (Fur$_{Vvbt1}$) is a 149 aminoacid protein with a similarity of 77% and 93% with Fur of *E. coli* (Fur$_{Ec}$) and *V. cholerae* (Fur$_{Vc}$) respectively. Fur$_{Vvbt1}$ is part of a complex network of regulation, only poorly characterized, where interacts with other transcriptional regulators. Thus, Fur$_{Vvbt1}$ is positively regulated by RpoS, as well as by itself, and represses directly the transcription of *V. harveyi* LuxR homolog, SmcR (Lee *et al.,* 2003; Lee *et al.,* 2007a; Kim *et al.,* 2013a). This fact suggests that Fur$_{Vvbt1}$ has an important role as a global regulator of gene expression at transcription level in *V. vulnificus*, regardless biotype and/or serovar.

Whole-genome based microarrays constitute a powerful tool to study the global transcriptomic response in bacteria. This technology has been widely used to find out the role of global transcriptional regulators in gene expression, as well as the influence of stimuli or environmental conditions in the transcriptomic response (Mueller *et al.,* 2009; Massé *et al.,* 2005; Jittawuttipoka *et al.,* 2010). In *V. cholerae* Mey and cols. (Mey *et al.,* 2005) defined the Fur$_{Vc}$ regulon by identifying genes up- or down-regulated in a *fur$_{Vc}$* mutant, and demonstrated that Fur$_{Vc}$ regulates a high number of genes belonging to different functional categories. Further, Alice and cols. (Alice *et al.,* 2008) performed a global transcriptomic analysis of *V. vulnificus* biotype 1 in response to different iron concentrations and defined condition-specific

transcriptomic profiles that included genes related to a wide variety of biological functions.

To further define the gene repertoire that is regulated by iron and Fur in the zoonotic serovar of *V. vulnificus*, we obtained a mutant in Fur$_{Vvbt2}$ and used DNA microarray technology to monitor the expression of the entire gene repertoire of the zoonotic serovar in response to iron. To this end, we first developed a specific Vvbt2serE-based microarray containing probes for all the ORF identified in the genome of the strain CECT4999. Global transcriptomic response was reconstructed by comparing the transcriptional profiles of the wild-type and *fur$_{Vvbt2}$* mutant strains in poor and rich iron conditions.

II.   MATERIAL AND METHODS

A.   Bacterial strains, growth media and conditions

Bacterial strains (Table 1) were routinely grown in LB-1/LBA-1 or CM9/CM9A (Sambrook and Russell, 2001). If necessary, ampicilin (100 µg/ml), chloramphenicol (20 µg/ml) or polymixin B (50 U/ml) were added to the media. To analyze the effect of different iron sources on growth, bacteria were grown in CM9-D (CM9 plus 20 µM dipyridil [Sigma]), CM9-Hm (0.1 µM bovine hemin [Sigma] plus 100 µM EDDA [Sigma]), CM9-Tf (40 µM iron-free human apo-transferrin [Sigma]). *V. vulnificus* strains were incubated at 28ºC and *E. coli* strains at 37ºC for 18-24 h. All the strains were stored in LB-1 plus glycerol (17%) at -80ºC.

Table 1. Bacterial strains and plasmids used in this study.

Designation	Description	Isolation source/ Reference
V. vulnificus		
CECT4999	Biotype 2 Serovar E	Diseased eel (Spain)
Δ*fur*	CECT4999 *fur*-defective mutant	This study
c*fur*	Δ*fur* complemented strain	This study
E. coli		
DH5α	Cloning strain	Invitrogen
s17-1λ*pir*	Strain containing the pCVD442 plasmid. *thi pro hsdR hsdM+ recA::RP4-2-Tc::Mu λpir Km^r Nal^r*	Simon *et al.* (1983)
Plasmids		
pGemT-easy	T/A Cloning vector, Amp^r	Promega
pIT009	Derivative of pJRD215 with the Sm^r gene between two *Xmn*I sites replaced by the multiple-cloning-site-containing *lacZ* gene cloned from pUC19	Lee *et al.* (2008)
pΔ*fur*	pGemT-easy with Δ*fur* in the MCS	This study
pIT*fur*	pIT009 with *fur* gene and promoter in MCS	This study

B.   DNA/RNA manipulation

1.   General technics

The general techniques of acid nucleic manipulation, PCR and qRT-PCR are detailed in Chapter 1, pages 71-74. Primers were designed from the genome of *V. vulnificus* YJ016 (Genebank: chromosome 1 BA000037, chromosome 2 BA000038 and plasmid pYJ016 AP005352) and the virulence plasmid pR99 (AM293858) (Table 2). RNA concentration and integrity were measured by 2100 Bioanalizer (Agilent), following the manufacturer's instructions. All samples presented a RNA Integrity Number (RIN) value higher than 9.

2.   Isolation of mutant and complemented strains

A *fur*$_{Vvbt2}$ insertion mutant was obtained as previously described (Shao and Hor, 2000) with slight modifications. Briefly, the regions of the chromosome corresponding to up-(1382 nt) and downstream (1329 nt) of *fur*$_{Vvbt2}$ were amplified using primer sets (Fur-1/Fur-2 and Fur-3/Fur-4) (Table 2) and cloned into the pGEMT-easy, carrying an in-frame deletion of the major part of the coding sequence. Chloramphenicol resistance marker was inserted at *Xba*I site of the cloned construction thus obtaining plasmid pΔ*fur*$_{Vvbt2}$. Plasmid was linearized by *Xmn*I digestion and introduced in *V. vulnificus* CECT4999 by natural transformation, as previously described (Meibom *et al*., 2005). Transformants were selected in Lb plates supplemented with chloramphenicol. To generate the complemented strain, c*fur*$_{Vvbt2}$, the entire *fur*$_{Vvbt2}$ gene and its promoter region was amplified from *V. vulnificus* CECT4999 with primers Fur-5/Fur-6 with a *Bam*HI restriction site added, and cloned into the *Bam*HI site of a recombinant plasmid, pIT009 (Lee *et al.,* 2008). The resultant plasmid (pIT *fur*$_{Vvbt2}$) was introduced into Δ*fur*$_{Vvbt2}$ by conjugation.

Table 2. Primers used in this study.

Mutant construction				
Primer	**Restriction site**	**Sequence**	**Product size(bp)**	**Utilization**
fur-1		GGTAAAGCGTGTCTTCGTGC	1382	Construction
fur-2	*Xba*I	CCTCTAGACAAGTGTGGCGATGGCTC		Construction
fur-3	*Xba*I	GCTCTAGACCCGTTGATGATCTGCCG	1329	Construction
fur-4		GTGTGGCTAGTGCTCTTCC		Construction
fur-cF	*Bam*HI	CTGGATCCGAGCGTATGGGTTACTTC	1060	Complementation
fur-cR	*Bam*HI	CTGGATCCGTTAAAGAGAAAATAC		Complementation

Microarray validation (qRT-PCR)			
Primer	**Sequence**	**Product size(bp)**	**Gene**
recA-F	CGCCAAAGGCAGAAATCG	59	Recombinase A
recA-R	ACGAGCTTGAAGACCCATGTG		
V1-F	AACGCCTTCCCCAATGC	54	2,3-DHBA-AMP ligase
V1-R	CAATCAAGCCTTCCGCCATA		
V2-F	CACCGAGTGCTGGAGTTGTTC	59	ABCt, ATP-binding protein
V2-R	TGTATACGCCTGTTGCGGATT		
V3-F	AGCAGCAACAAATGGCGATA	59	Polar flagellar sheath proteinA
V3-R	CCTGCAGTCGCGATCGTT		
V4-F	CGCGTAGGCGAAACACTGAT	62	Carbon storage regulator
V4-R	GCCTTTAACACCCAGTACCGTTA		
V5-F	AATCCGCGCACTCAGCAT	54	Transketolase
V5-R	GCCTGGATGGCCTGAGTTT		
V6-F	AATCTGGGCAACAGAATCTATGG	63	Pyruvate formate-lyase
V6-R	TGAACGCGTTACTAGCGTACGA		
V7-F	AAGGCATCCCAAATCTGCAA	59	Bacterioferritin
V7-R	TTTCTTGGGTATCTTCGCCAAT		
V8-F	TTTGCCGCCATCAAACAA	53	Catechol ABC transporter
V8-R	GATGGTGAGCGCATCCACTT		
V9-F	TCGCTGGGAAGGCCATATT	55	33 kDa chaperonin
V9-R	CTTTGCCCATCATGTCGTGTA		
V10-F	AGCAGCAACAAATGGCGATA	59	Polar flagellar sheath proteinA
V10-R	CCTGCAGTCGCGATCGTT		
V11-F	CAAAACGCAAAAGTGAACAAGAA	57	DNA-directed RNApol
V11-R	CCCGGCGTATTGCTGTTG		
V12-F	TCTCTTCTTTTGGCTCAACGTTT	60	TcuB
V12-R	TCCAATCCTCCCCCTTCCT		

C.   Microarray analysis

1.   Microarray design

The VvBt2SerE-specific gene expression microarray (8x15K) slides were custom designed with eArray software (Agilent technologies), following MIAME guidelines for array design (Brazma *et al.,* 2001). The predicted annotated ORF's of CECT4999 strain genome (unpublished data) was used for the probes design. The arrays contained in total 4553 probes of 60-oligonucleotide length. These probes were distributed in 3 probes per target (13890) with an e-value of 0.0 and the rest were filled with internal control probes of Agilent. Settings used were based on the following: Base composition methodology, best probe methodology, and design with 3' bias.

2.   Labelling and hybridization

General procedures to obtain labeled cRNA were performed as described in protocols of the kit "One-Color Microarray-Based Gene Expression Analysis: Low Input Quick Amp Labeling" (Agilent).

First step consisted in preparation of One-Color Spike Mix, a mix of ten *in vitro* transcripts in predetermined ratios that are processed in parallel with the rest of the samples and allows researchers to efficiently monitor microarray workflow for linearity, sensitivity and accuracy after hybridization onto Agilent microarray control probes.

To obtain cDNA, 200 μg of sample RNA (template) were mixed with 200 ng of T7N9 primers, a random nonamers that amplify all the RNA (Moreno-Paz and Parro, 2006). Resultant cDNA was subjected to a transcription reaction to finally obtain cRNA labeled with cyanine 3 dye (Cy3). Purification and quantification of labeled/amplified cRNA in Nanodrop ND-2000 was carried out as described in manufacturer's protocols.

Cy3-labeled cRNA was hybridized with array slides as detailed in manufacturer's instructions (Agilent); labeled cRNA was subjected to a fragmentation reaction for 30 minutes and samples were incubated on ice for 1 minute to stop the reaction; labeled cRNA makes a better target for oligo arrays once it has been fragmented to an optimal size of 50–200 bases long, thus, the structures of the fragmented targets are less complex, which helps improve their specificity and raises the average feature signal intensity on the microarray.

For hybridization incubation, samples were dispensed onto the gasket well on the slides and were placed in a hybridization oven with rotation at 10 rpm at 65ºC for 17 hours. Washing steps of the slides were carried out as described in manufacturer's protocol and scanning was performed with an Axon Scanner 4000B.

3. Microarray validation by qRT-PCR

To validate the microarray expression results, the same samples used in microarray analysis were analysed by qRT-PCR (described in Chapter 1 page 73) to calculate the expression of 12 selected genes at transcription level. Primers specific to the housekeeping gene *recA* and tested genes were used to amplify DNA fragments of about 60 bp (Table 2). The tested genes were selected on the basis of their transcriptional activity classified in **induced** (fold change ≥2), **repressed** (fold change ≤-2) and **invariable expression** (2>fold change>-2), in both Δ *fur$_{Vvbt2}$* and presence of transferrin conditions.

D.   Phenotypic characterization of furVvbt2 mutant

1. Motility assay

A volume of 5 μl of a bacterial suspension in PBS-1 (10^9 CFU/ml) from exponential phase cultures (6 h) in CM9 was spotted on MA (Motility Agar; tryptone 1%, yeast extract 0,5%, NaCl 1% and agar 0,3%), MA-Fe (MA plus 100 μM FeCl$_3$) or

MA-D (MA plus 20 µM dipyridil) plates and diameter of halos was measured by triplicate. Microscopic observations of bacterial suspension were made in a Nikon Phase-Contrast Microscope.

2. Chemotaxis assay

Chemotaxis assay was performed as described previously (Larsen *et al.,* 2001). Capillary tubes (5-µl pre-calibrated pipettes; Vitrex) were filled with EM (eel mucus) or ChB (chemotaxis buffer; PBS + 0.01 mM EDTA) and were introduced up to 1 cm in eppendorfs containing 0.5 ml of a bacterial suspension in ChB (10^8 CFU/ml) from exponential-phase-growth cultures (6h) in CM9 or CM9-D. Eppendorfs plus fixed capillary tubes were incubated for 30 min at 28ºC and bacterial numbers both inside and outside the capillary tubes were estimated by drop plating on TSA-1 plates (Hoben and Somasegaran, 1982). The chemotactic response (CR) was expressed as the ratio between bacterial counts in EM-capillarity tubes *vs* ChB-capillarity tube.

3. Bacterial attachment

To determine the attachament degree to a chitin surface, a 50-ml falcon flask containing 10 ml of artificial sea water (ASW; KCl 0.067%, $CaCl_2 2H_2O$ 0.136%, $MgCl_2 6H_2O$ 0.466%, $MgSO_4 7H_2O$ 0.629%, $NaHCO_3$ 0.018% and CaCl 2.47%) (supplemented or not with dipyridil at 20 µM) plus 0.5 gr of PBS-washed and autoclaved crab shell was inoculated with overnight cultures of the wild-type and its derivative strains in CM9 or CM9-D (ratio of 1:100, vol/vol) and incubated at 17ºC with shaking (70 rpm). DNA was extracted at 0, 24 and 48h post-incubation after washing crab shells twice with PBS-1 by recovering bacteria with Mili-Q water, vortexing and heating at 100ºC for 10 min. DNA was quantified by quantitative PCR (qPCR) by using primers specific for *recA* housekeeping gene (recA-F/recA-R) (Table 2).

4. Growth in plasma, hemin and apo-transferrin

Microtitter plates of 96-wells containing fresh eel and human plasma (200 μl/well) were inoculated with stationary-phase bacteria at a ratio 1:100 (vol/vol) and were incubated for 10 h at 28 or 37ºC (HP) with shaking (200 rpm). If necessary, the bactericidal (complement) or bacteriostatic (Tf) activity of plasma was abolished by heating it at 56ºC for 30 min (Amaro *et al.*, 1997) or supplementing it with 100 μM of $FeCl_3$, respectively. Plasma was obtained as described in Chapter 1, page 78. OD_{600} was measured at 1 h-intervals and growth curves were constructed and statistically compared.

5. Minimal inhibitory concentration (MIC) of iron chelators, microcide peptides and saponin (a surfactant from plants)

MICs were determined in 96-well microtitter plates containing CM9 (200 μl per well) supplemented with different concentrations of polymyxin B sulfate (10 to $8x10^3$ U/ml; Sigma), lysozyme (10 to 10^3 μg/ml; Sigma), dipyridil (10 to 200 μM; Sigma), apo-transferrin (10 to 100 μM; Sigma) or saponin (100 μg/ml; Sigma). Plates were inoculated with overnight cultures in CM9 in a ratio 1:100 (vol/vol) and were incubated for 30 min (saponin) or 24 h at 28°C with shaking (160 rpm). The MIC endpoint was defined as the lowest substrate concentration at which there was not visible growth except in case of saponin-containig paltes, in which a culturable count on CM9A was performed (Hoben and Somasegaran, 1982) at 5, 10, 15, 20 and 30 min post-incubation.

6. Siderophore detection

Siderophore production was determined as described in Chapter 2 page 113.

7.   Resistance to acid, heat and cold shocks

For cold-shock resistance determination, overnight cultures in CM9 or CM9-D (28ºC, 160 rpm) or were maintained at 28ºC (control) or were transferred to a 4ºC-cold chamber where were additionally incubated for 10 days with agitation (160 rpm). For acid- and heat-shock resistance determination, bacteria from overnight cultures (at 28ºC, both shocks, or 37ºC, only heat-shock) were washed in PBS-1 and inoculated in tubes containing 5 ml of PBS-1 or PBS1-pH5 (only acid-shock), both supplemented or not with dipyridil (20 µM), at a ratio of 10^5 CFU/ml. Tubes were incubated at 28ºC (both shocks) or 41ºC (only heat-shock) with shaking (160 rpm) for 180 min (acid-shock) or 10 h (heat-shock). In all cases, culturable bacteria numbers were estimated by drop plating (Hoben and Somasegaran, 1982) on CM9A at intervals of 1 day (cold-shock), 30 min (acid-shock) or 1 h (heat-shock).

8.   Proteolytic, hemolytic and chitinase activity

The protease activity of ECPs (crude extracts of toxins and exoenzymes, also called extracellular products, ECP), obtained as described in Chapter 1 page 71 from overnight cultures on CM9A or CM9A-D, was estimated according to Miyoshi and cols. (Miyoshi *et al.*, 2002). Briefly, eppendorfs containing 0.5 ml of ECP plus 0.5 ml of azocasein solution (2 mg/ml in 50mM Tris-HCl buffer, pH 8.0) were incubated in a 30ºC-thermostated bath for 15 min. Then, 0.5 ml of 5% trichloroacetic acid were added to stop proteolysis, precipitates were eliminated by centrifugation (13.000 rpm, 5 min), and a volume of 0.5 ml of the supernatant was mixed with 0.5 ml of NaOH 0.5M and Abs_{440} was measured. One protease unit (PU) was defined as the amount of the sample hydrolyzing 1 mg of the substrate in 1 ml, and was calculated as follows:

$$UP = 1000 \times (A440/15) \times \text{inverse dilution}$$

Hemolytic activity against bovine erythrocytes of bacterial cells and ECP (from cultures in CM9 or CM9-D) was measured as detailed in chapter 1 page 77.

Bacterial growth from chitin as the sole carbon source was determined as an indirect estimation of chitinase activity. To this end, bacteria from overnight CM9 or CM9-D cultures (28ºC, 160 rpm) were washed twice in PBS-1 and inoculated (10^6 CFU/ml) in ASW-chitin (Artificial sea water plus 1% [vol/vol] colloidal chitin [Sigma]), supplemented or not with dipyridil 20µM. Bacteria were incubated at 28ºC with shaking (160 rpm) and culturable counts were performed at 0, 3, 7 and 14 days post-incubation onto CM9A by drop plating (Hoben and Somasegaran, 1982).

9.   LPS and capsule

Crude fractions of LPS and capsule were obtained from overnight CM9 or CM9-D as described by Hitchcock and Brown (1983). Briefly, bacteria from 1 ml of culture were washed twice with PBS-1, dispersed in 50 µl of FSB (Final Sample Buffer; Tris-HCl 0.065M, pH 6.8, SDS 2%, Glicerol 10%, Bromophenol Blue 0.001% and 2-mercaptoethanol 5%) and lysed by boiling at 100ºC during 10 min. Proteins were degraded with proteinase K (Sigma Aldrich), by adding 20 µl of a solution of 2.5 mg/ml in FSB and incubating 1 h at 65ºC. The polysaccharyde concentration was determined with ProQ Emerald 300 staining for glycoproteins (Invitrogen) by following the manufacturer's instructions. LPS and capsule antigens were separated by SDS-PAGE (Laemmli, 1970) in discontinuous gels (4% stacking gel, 10% separating gel), transferred to a PVDF membrane (0.2 µm, Bio-Rad) at 100V for 1 h in Tris-glycine-methanol transfer buffer (25 mM Tris, 192 mM glycine [pH 8.3], 20% [vol/vol] methanol) (Towbin *et al.*, 1979), immunostained with serovar E-specific sera (Amaro *et al.,* 1992) diluted 1:3000 plus anti-rabbit IgG HRP-conjugated sera diluted 1:5000 (Sigma Aldrich), and revealed with Immobilon Western Chemiluminescent HRP Substrate (Millipore) in a Image Quant™ LAS4000mini biomolecular imager (GE healthcare).

10.   Virulence and colonization/invasion

Animal maintenance, virulence degree and colonization/invasion assays were performed as described in Chapter 1 page 80-81.

E.   Statistical analysis

All the experiments were performed by triplicate and the significance of the differences was tested by using the unpaired Student's t-test with a $p < 0.05$.

III.   RESULTS

A.   Microarray analysis

A very high number of genes changed their expression level when fur_{Vvbt2} was mutated or when iron was sequestered from the medium by apo-transferrin (Figure 1). Thus, 1791 genes (39.5% of the genome) were differentially expressed by the fur_{Vvbt2} mutant, from which 969 (54.1%) resulted to be induced and 822 (55.9%) repressed, and 1318 (29% of the genome) were regulated by iron, 595 (45.14%) up-regulated and 723 (54.86%) down-regulated (Figure 1). Interestingly, a 62% of plasmidic genes seems to be regulated by Fur $_{Vvbt2}$/Iron.
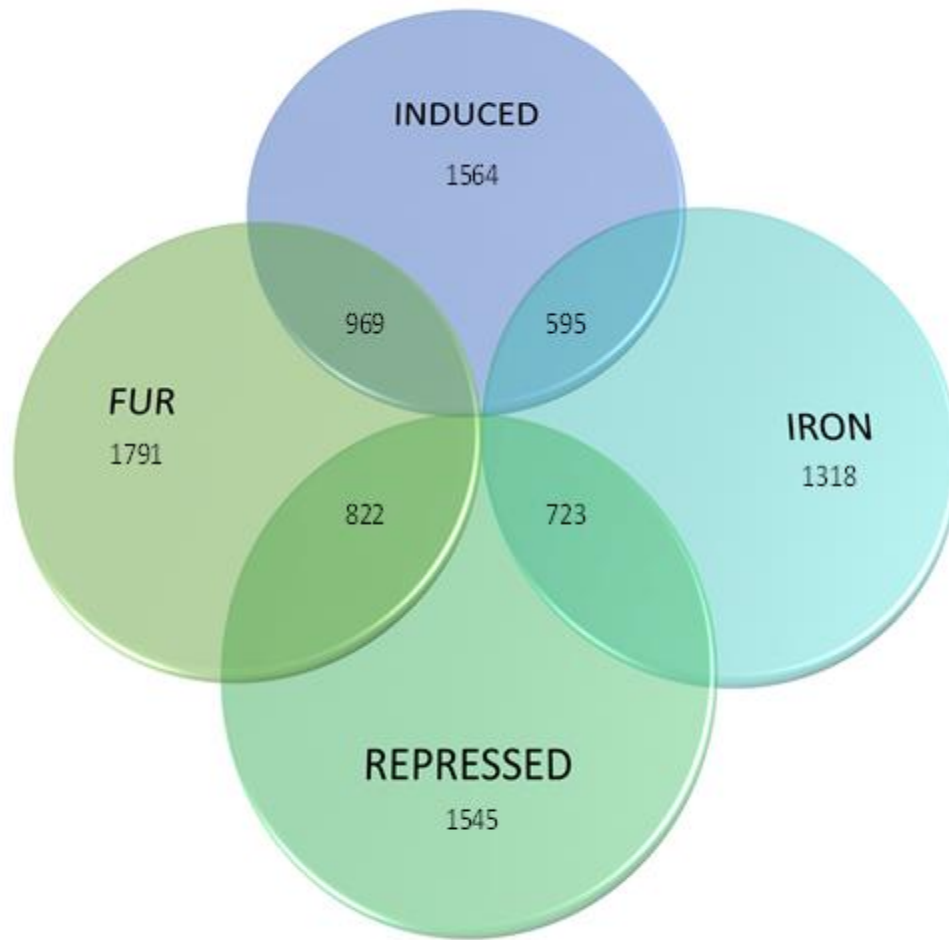
Figure 1. **Diagrams representing the genes induced/represed by Fur$_{Vvbt2}$ and iron.**

To validate the microarray data, the expression level of selected genes was also determined by qRT-PCR. For the selection, the genes were classified in Fur$_{Vvbt2}$-*vs* iron-regulated and within each category in up- (fold change ≥2, p-value $\leq$ 0.01.), down- (fold change ≤-2, p-value $\leq$ 0.01.) or "non"-regulated (2>fold change>-2, p-value $\leq$ 0.01). The selected genes and their expression levels evaluated from the same sample by both microarray and qRT-PCR analysis are presented in Table 3. A good correlation was found between both kinds of data, which validated the microarray analysis.

Table 3. Comparison of mRNA levels in *V. vulnificus* array and qRT-PCR.

Sample	Gene	Fold Change[a]	
		Array	**qRT-PCR**
Δ*fur* in CM9	2,3-dihydroxybenzoate-AMP ligase	21.9 (++)	13.92 (++)
	ABC transporter, ATP-binding protein	11.78 (++)	15.03 (++)
	54K polar flagellar sheath protein A	- 34.17 (---)	-5 (-)
	Carbon storage regulator	- 24.26 (--)	-2.7 (-)
	Transketolase	1.05 (=)	1.13 (=)
	Pyruvate formate-lyase	1.04 (=)	1.14 (=)
CECT4999 in CM9-Tf	Bacterioferritin	45.19 (+++)	24.33 (++)
	Catechol ABC transporter, substrate-binding protein	41.18 (+++)	7.51 (+)
	33 kDa chaperonin	- 17.36 (--)	-2.63 (-)
	54K polar flagellar sheath protein A	- 55.3 (---)	-11.11 (--)
	DNA-directed RNA polymerase, beta' subunit	1.3 (=)	1.18 (=)
	TcuB	1.19 (=)	1.16 (=)

[a] Qualitative classification: =, $-2<X<2$; +, $2 \leq X<10$; ++, $10 \leq X<25$; +++, $25 \leq X$; -, $-10<X \leq -2$; --, $-25<X \leq -10$; ---, $X \leq -25$.

Classically, the Fur protein has been thought to act coordinately with iron as a repressor, but in the last years has been proved that the global regulator Fur can control gene expression without interacting with iron and also acting as an activator (Carpenter *et al.,* 2009). In consequence, microarray results were analyzed in depth and variable-expression genes were further classified into eight categories, which were not-equally represented (Figure 2). The differentially expressed genes classified by categories are listed in appendix III.
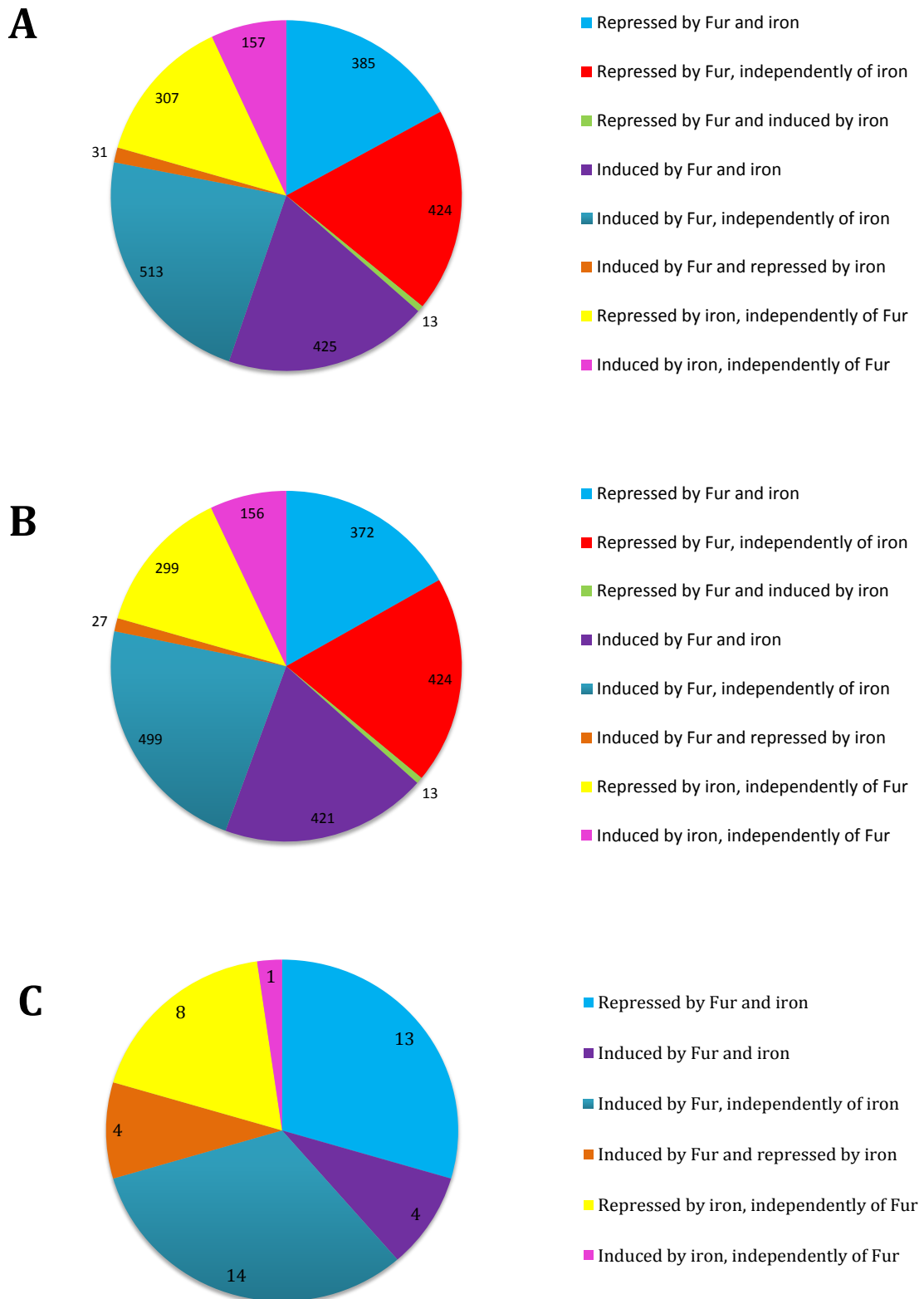
Figure 2. **Number of genes regulated by Fur and iron**. A) All genes, B) chromosomal genes and C) plasmidic genes.

We focused our research on putative virulence and transcription-related genes since they could be of some help in disentangling the role played by Fur_{Vvbt2} and iron in the pathogenesis of this zoonotic pathogen. In table 4, genes induced, repressed or non-altered are represented with green, red and white color, respectively.

TOXINS AND EXOENZYMES

Table 4a. Selected genes under control of Fur_{Vvbt2} and/or iron: Toxins and exoenzymes.

GENE CATEGORY AND NAME	FUR	IRON
TOXINS AND EXOENZYMES		
HEMOLYSINS		
21 kDa hemolysin precursor	green	green
Hemolysin III homolog	green	
Hemolysins and related proteins containing CBS domains	red	red
Putative hemolysin	red	red
Putative hemolysin		red
Thermolabile hemolysin precursor	red	red
PROTEASES		
Exported zinc metalloprotease YfgC precursor	red	red
Membrane-associated zinc metalloprotease	green	
Protease II	red	red
Protease IV	red	red
Protease-related protein	red	red
Putative protease	red	
Putative protease La homolog		green
Tail-specific protease precursor	red	red
CYTOLYSINS AND RTX		
Cytolysin precursor		red
Cytolysin secretion protein		red
Putative RTX toxin	green	green
RTX toxins and related Ca^{2+}-binding proteins		red
RTX transporter		red
RtxC	red	
CHITIN-RELATED PROTEINS		
Chitin binding protein	red	red
Chitinase	green	
Chitinase	green	

A few genes for toxins (putative hemolysins) and proteases seem to be under Fur$_{Vvbt2}$ / iron control although none of them corresponds to the major cytolysin/hemolysin, RtxA1$_3$ and VvhA, and proteases, VvpE of *V. vulnificus* (Lee *et al.,* 2012; Lee *et al.,* 2004; Shao and Hor, 2000). Among the activated genes, it should be highlighted the hemolysin III*,* described in the biotype 1 as a virulence gene whose mutation causes attenuation in virulence for mice (Chen *et al.,* 2004a), two chitinases and a gen related with a putative RTX toxin, homologous to a toxin present in *E. coli* and *Neisseria* sp., without a clear role in virulence (Forman *et al.,* 2003). Regarding the repressed genes, *rtxC* is part of the operon *rtx* of *V. vulnificus* and encodes an enzyme that supposedly activates post-transcriptionally the main virulence factor in the biotype 2, the MARTX type III, and *yfgC*, homolog to *bepA* of *E. coli,* is a gene related to OM integrity that promotes either protein inclusion or exclusion by proteolytic degradation when they are incorrectly assembled (Narita *et al,* 2013).

IRON ACQUISITION SYSTEMS

Table 4b. Selected genes under control of Fur$_{Vvbt2}$ and/or iron: Iron acquisition systems.

GENE CATEGORY AND NAME	FUR	IRON
IRON ACQUISITION SYSTEMS		
HEMIN/HEMOGLOBIN		
Hemin receptor HupA	🟥	🟥
Hemin receptor HutR	🟥	🟥
Hemin transport, ABC transporter, ATPase component	🟥	🟥
Hemin transport, ABC transporter, permease component	🟥	🟥
Hemin transport, ABC transporter, periplasmic component	🟥	🟥
Putative heme iron utilization protein	🟥	🟥
Pyridoxamine 5'-phosphate oxidase (heme iron utilization protein)		🟥
FERRIC/FERROUS IRON UPTAKE		
Ferric iron ABC transporter, iron-binding protein	🟥	🟥
Ferric iron ABC transporter, permease component		🟥
Ferrous iron transport protein A	🟥	🟥
Ferrous iron transport protein B	🟥	🟥
Ferrous iron transport protein C	🟥	🟥
FERRIREDUCTASES		
Predicted ferric reductase	🟩	

IRON STORAGE		
Bacterioferritin	Red	Red
Bacterioferritin-associated ferredoxin	Red	Red

SIDEROPHORES BIOSYNTHESIS AND TRANSPORT		
ABC-type Fe^{3+}-hydroxamate transport system, periplasmic component	Green	Green
ABC-type metal ion transport system, periplasmic component	Green	Green
Anthranilate synthase, amidotransferase component		Red
Anthranilate synthase, aminase component	Green	
Catechol siderophore ABC transporter, substrate-binding protein	Red	Red
Ferric aerobactin ABC transporter, ATPase component	Red	Red
Ferric aerobactin ABC transporter, periplasmic component	Red	Red
Ferric aerobactin ABC transporter, permease component	Red	Red
Ferric aerobactin siderophore receptor IutA		Red
Ferric aerobactin; hypothetical protein in aerobactin cluster		Red
Ferric siderophore transport system, biopolymer transport protein ExbD/TolR	Red	Red
Ferric siderophore transport system, biopolymer transport protein ExbD1	Red	Red
Ferric siderophore transport system, biopolymer transport protein ExbB	Red	Red
Ferric siderophore transport system, biopolymer transport protein ExbB	Red	Red
Ferric siderophore transport system, periplasmic binding protein TonB1	Red	Red
Ferric siderophore transport system, periplasmic binding protein TonB2	Red	Red
Ferric siderophore transport system, periplasmic binding protein TonB3	Green	
Ferric vibriobactin, enterobactin transport system, ATP-binding protein	Red	Red
Ferric vibriobactin, enterobactin transport system, permease protein VctD	Red	Red
Ferric vibriobactin, enterobactin transport system, permease protein VctG	Red	Red
Ferrichrome-iron receptor	Red	
Isochorismatase	Red	Red
Isochorismate synthase	Red	Red
Isochorismate pyruvate-lyase		Red
Non-ribosomal peptide synthetase modules	Red	Red
Non-ribosomal peptide synthetase modules	Red	Red
Phosphopantetheinyl transferase component		Red
Outer membrane receptor for ferrienterochelin	Green	Green
pR99 Vep20	Red	Red
Vulnibactin utilization protein VuuB	Red	Red

As expected, the genes involved in iron acquisition were putatively repressed by Fur$_{Vvbt2}$ and iron with very few exceptions (Table 4b). Thus we identified:

i) *heme-uptake related genes*: among them, *hupA* and *hutR*, which encode hemin receptors (characterized in chapter 2), a set of genes involved in hemin transport and two putative heme utilization genes, one of them only under iron control.

ii) *siderophore- and transferrin-related iron utilization:* We identified a series of genes for vulnibactin biosynthesis (previous steps: genes for two subunits of the

anthranilate synthase -one repressed by iron and the other activated by Fur_{Vvbt2}- involved in aminoacid biosynthesis; specific steps: genes belonging to the vulnibactin biosynthetic operon such as those for three enzymes related to the metabolism of isochorismate, 2 non-ribosomal peptide synthetases...) and for vulnibactin transport (*exbD*, *exbD1*, two copies of *exbB* and the three *tonB* systems, of which *tonB3* resulted to be induced by Fur_{Vvbt2}, accordingly with the results of Alice *et al.,* 2008). In addition we found series of genes for the uptake of exogenous siderophores, such as genes for a ferrienterochelin –or ferrienterobactin- receptor, for two periplasmic components of ABC transporters, one for hydroxamates and the other for metal ions (all the three genes being up-regulated), together with the cluster for aerobactin utilization characterized in biotype 1 (genes for ATPase, periplasmic and permease components of the ABC transporter, as well as the aerobactin receptor *iutA* and a hypothetical protein), three components of a vibriobactin-enterobactin transport system and a ferrichrome receptor. Finally, the plasmid gene *vep20,* described in chapter 2, which encodes a putative transferrin binding protein was also detected.

iii) *ferric and ferrous iron transport related genes*: these genes have been recently characterized in *V. cholerae* (Weaver *et al.,* 2013) and were repressed by Fur_{Vvbt2} and iron (except the permease component of the ABC transporter of ferric iron, that was only repressed by iron).

iv) a predicted ferri-reductase (induced by iron) and 2 bacterioferritins for iron storage.

RESISTANCES

Table 4c. Selected genes under control of Fur$_{Vvbt2}$ and/or iron: Resistances.

GENE CATEGORY AND NAME	FUR	IRON
RESISTANCES		
RESISTANCE TO COLD SHOCK		
Cold shock protein CspA	🟥	🟥
Cold shock protein CspD	🟩	
Cold shock protein CspE	🟥	🟥
Cold shock protein CspG	🟥	🟥
Cold shock DEAD-box protein A	🟥	
RESISTANCE TO HEAT SHOCK		
16 kDa heat shock protein A		🟩
Heat shock protein GrpE	🟩	
Heat shock protein Hsp33	🟩	🟩
Heat shock protein Hsp60 (GroEL family)	🟩	🟩
Heat shock protein Hsp60 (GroEL family)	🟩	🟩
Heat shock protein Hsp60 (GroES family)	🟩	🟩
Heat shock protein Hsp60 (GroES family)	🟩	🟩
Heat shock protein HspA	🟩	🟩
Heat shock protein YciM	🟩	🟩
Possible protease SohB		🟩
Probable protease HtpX homolog	🟩	🟩
Ribosome-associated heat shock protein	🟩	🟩
RESISTANCE TO OXITADIVE AND NITROSATIVE STRESS		
Alkyl hydroperoxide reductase	🟥	🟥
Alkyl hydroperoxide reductase protein C	🟩	🟩
Catalase	🟩	🟩
Glutathione peroxidase	🟥	🟥
Manganese superoxide dismutase	🟥	🟥
OsmC/Ohr family protein	🟩	
Tiol peroxidase, Bcp-type	🟥	🟥
YaaA protein	🟩	
	🟩	
Nitrite transporter	🟩	🟩
Nitrite-sensitive transcriptional repressor NsrR	🟩	🟩
Nitrogen regulatory protein	🟩	🟩
NnrS protein involved in response to nitric oxide	🟩	🟥
Periplasmic nitrate reductase precursor		🟥
Periplasmic nitrite reductase NapD	🟩	
Periplasmic nitrite reductase NapE	🟩	🟩
Periplasmic nitrite reductase NapF	🟩	
Periplasmic nitrite reductase NapH	🟩	
RESISTANCE TO MICROCIDE COMPOUNDS		
ABC-type multidrug transport system, ATPase component	🟩	🟩
ABC-type multidrug transport system, permease component	🟩	🟩
ABC-type multidrug transport system, permease component	🟥	

Protein	Col 1	Col 2
Acriflavine resistance protein	red	red
Acriflavine resistance protein	red	red
Membrane component of multidrug resistance system		green
Membrane fusion component of multidrug resistance system	red	red
Membrane fusion protein of RND[1] family multidrug efflux pump	red	
Membrane fusion protein of RND family multidrug efflux pump	green	
Multidrug resistance protein 2	green	
Multidrug resistance protein A	green	green
Multidrug resistance protein D	green	
Multidrug resistance protein SanA	green	green
Multiple antibiotic resistance protein MarC	red	
Na$^+$-driven multidrug efflux pump	green	
Outer membrane protein OmpU	red	red
Permease of the drug/metabolite transporter (DMT[2]) superfamily	red	red
Permease of the drug/metabolite transporter (DMT) superfamily	green	
Permease of the drug/metabolite transporter (DMT) superfamily	green	
Permease of the drug/metabolite transporter (DMT) superfamily	green	green
Permease of the drug/metabolite transporter (DMT) superfamily		red
Permease of the drug/metabolite transporter (DMT) superfamily		red
Putative multidrug resistance protein	green	
Tellurite resistance protein	green	green
TldD protein, part of proposed TldE/TldD proteolytic complex	red	red

RESISTANCE TO PLASMA AND OTHER STRESS CONDITIONS

Protein	Col 1	Col 2
KtrA		green
pR99 Vep07	red	red
SOS response repressor and protease LexA	green	
Membrane stress response protease DegS		red
Sensing protein RspA	green	green
Starvation lipoprotein Slp	green	green
Starvation protein A		green
Survival protein SurE	red	red
Universal stress protein A	green	
Universal stress protein B	green	green
Universal stress protein family 8	green	

BIOFILM FORMATION AND RESISTANCE TO PLASMA
LPS and Lipid A biosynthesis and transport

Protein	Col 1	Col 2
Lipid A biosynthesis (KDO[3]) 2-(lauroyl)-lipid IVa[4] acyltransferase	red	
Lipid A biosynthesis UDP[5]-2,3-diacylglucosamine hydrolase	red	red
Lipid A core-O-antigen ligase	red	red
Lipid A export ATP-binding/permease protein MsbA	red	
Lipid A-disaccharide synthase	red	
Lipopolysaccharide ABC transporter, ATP-binding protein LptB	red	
Lipopolysaccharide biosynthesis protein RffC	red	red
LptA, protein essential for LPS transport across the periplasm	red	
O-antigen flippase Wzx	green	
Putative LPS biosynthesis protein	red	red
Putative LPS biosynthesis protein	red	red

Polysaccharide capsule

Protein	Col 1	Col 2
Capsular polysaccharide biosynthesis protein CapD	red	red
Capsular polysaccharide synthesis enzyme CpsA, sugar transferase	green	

Capsular polysaccharide synthesis enzyme CpsB	🟩	🟩
Capsular polysaccharide synthesis enzyme CpsC, polysaccharide export	🟩	
Capsular polysaccharide synthesis enzyme CpsD	🟩	
Capsular polysaccharide synthesis enzyme CpsG, Lipid A core - O-antigen ligase	🟩	
Capsular polysaccharide synthesis enzyme CpsI, glycosyltransferase	🟩	
Polysaccharide biosynthesis chain length regulator SypO	🟩	🟩

[1] RND: Resistance nodulation-cell division
[2] DMT: Drug metabolite transporter
[3] KDO: keto-deoxyoctulosonate
[4] IVa: distinct form of Lipid A
[5] UDP: Uridine diphosphate

As shown in the microarray results, many genes related with stress conditions resulted to be regulated by Fur$_{Vvbt2}$ and/or iron (Table 4c). The following functional groups were established:

Cold shock-resistance genes*.* Most of them except one (*cspD)* repressed by Fur$_{Vvbt2}$ and iron, three of them belong to the *csp* operon, previously characterized in *V. vulnificus* (Limthammahisorn *et al.,* 2008; Wood and Arias, 2011)*,* and the other is involved in the optimal cell growth at low temperature in *E. coli* (Jones *et al.,* 1996).

Heat shock-resistance genes. All of them positively regulated by Fur$_{Vvbt2}$ and/or iron. Among them, *hsp33,* several *hsp60,* a gene for a 16KDa HSP A, all of them related with protein-protein interactions (folding, establishment of proper-protein conformation [shape] and prevention of unwanted protein aggregation) in several bacteria (Borges y Ramos, 2005), *htpX*, related with a zinc-dependent endoprotease, involved in proteolytic quality control to prevent membrane malfolding and misassembling in *E. coli* (Sakoh *et al.,* 2005)*, grpE*, related to DNA replication at 42ºC in *E. coli* (Wu *et al.,* 1996), *hspA* related with the production of polyhydroxyalkanoic acid- based biopolymers in *E. coli*. (Tessmer *et al.,* 2007) and *sohB*, involved in the suppression of the temperature-sensitive phenotype and in cell viability at high temperatures also in *E. coli* (Baird *et al.,* 1991).

Oxidative stress-resistance genes. This group is formed by four induced and four repressed genes homologous to genes whose functionality has been probed in *E. coli*. Thus, the repressed genes would be related with enzymes that reduce organic or inorganic hydroperoxide (Chelikani *et al.,* 2004; Bhabak and Mugesh *et al.,* 2010; Seaver and Imlay, 2001) and the activated genes with a variety of proteins and functions such as an osmotically inducible protein (*osmC*), a stress-induced protein involved in organic hydroperoxide detoxification (Lesniak *et al.,* 2003), a protein (*yaaA*) that reduces hydroperoxide toxicity by diminishing the amount of intracellular unincorporated iron (Liu *et al.,* 2011), and, finally, a manganese-dependent superoxide dismutase, *sodA* that is involved not only in the dismutation of superoxide (O^{2-}) into oxygen and hydrogen peroxide, but also in resistance to acid stress (Kim *et al.,* 2005).

Microcide peptide- and bile-salts resistance genes. Most of the genes of this group were annotated as hypothetical proteins, belonging to a 2 superfamilies of proteins, the RND (resistance nodulation-cell division) and the DMT (drug metabolite transporter). The first one, RDN, comprises efflux pumps involved in the active transport of several compounds, including drugs, and the second one, DMT, is subdivided in a high number of subfamilies generally involved in mechanisms for drug resistance. Within this group are up-regulated and down-regulated genes. <u>Activated genes</u>: genes for ABC transporters (permease and ATPase), membrane component and membrane fusion proteins of RND superfamily, permeases of DMT superfamily, multidrug efflux pumps, several multidrug resistance proteins, and one protein of resistance to tellurite (a bile salt). <u>Repressed genes</u>: genes for one ABC transporter (permease), two membrane fusion proteins, one of them of RND superfamily, two proteins of resistance to acriflavine (antiseptic) and permeases of DMT superfamily. In addition, *marC* annotated as a multiple antibiotic resistance protein, whose real function is under discussion (McDermott *et al.,* 2008), the TldE/TldD proteolytic complex, that in *E. coli* is involved in the processing of microcin B17 and CcdA (peptide antibiotics produced by *E. coli*) (Allali *et al.,* 2002), and, finally, *ompU*, that has a fibronectin-binding function in *V. vulnificus* and an adhesion function in *V. cholerae* as well as is involved in resistance to antimicrobial peptides and bile-salts (Mathur and Waldor, 2004; Goo *et al.,* 2006).

Plasma resistance. Two genes related with resistance to plasma were identified, one of them *ktrA* is induced by iron and encodes a potassium pump *of V. vulnificus* involved in resistance to human plasma, protamine and polymixin B (Chen *et al.,* 2004b). The other gene is the plasmidic gene *vep07,* which encodes an OM lipoprotein that confers specific resistance to eel plasma and that is repressed by Fur$_{Vvbt2}$ and iron (our unpublished results).

Resistance to Nitric oxide. Several genes putatively involved in the detoxification process of nitric oxide are induced by Fur$_{Vvbt2}$ and/or iron, including the *napD, napE, napF* and *napH* genes that codifies for periplasmic nitrite reductases, and two important transcriptional regulators, *nsrR* and *nnrS* (Stewart *et al.,* 2009; Honisch and Zumft, 2003; Bodenmiller and Spiro, 2006).

Resistance to other stress conditions. A series of genes putatively involved in other stress conditions are repressed by Fur$_{Vvbt2}$ and/or iron, like *degS*, which encodes a membrane protease that activates a sigma factor related with restoration of membrane integrity (Chatterjee and Chowdhury, 2013), or *surE,* related to stationary phase survival and other stress-conditions in *E. coli* (Mura *et al.,* 2003). Other genes were activated by Fur$_{Vvbt2}$ and/or iron, like *lexA*, which encodes the SOS response repressor (Kimsey and Waldor, 2009), a series of genes encoding "universal stress proteins" related to survival in stationary phase, and other stress conditions (DNA damage) (Siegele, 2005), and several genes encoding starvation proteins (Slp, related to acid resistance [Masuda and Church, 2002] and starvation proteins [Groat *et al.,* 1986]), as well as *rspA*, which encodes a sensing protein related to stress (Sakihama *et al.,* 2012).

External envelopes. Interestingly, genes involved on Lipid A, LPS and O-antigen, as well as on capsular polysaccharide biosynthesis were identified being mainly repressed or induced, respectively, by Fur$_{Vvbt2}$ and/or iron. As explained in the introductions section, the O-antigen and capsule are clearly related to resistance to plasma and phagocytosis (Wright *et al.,* 1990; Valiente *et al.,* 2008b). The fact that their biosynthesis is regulated so clearly by Fur$_{Vvbt2}$ and iron is an evidence of the relevant role that these components have in *V. vulnificus* pathogenicity.

MOTILITY

Table 4d. Selected genes under control of Fur$_{Vvbt2}$ and/or iron: Motility.

GENE CATEGORY AND NAME	FUR	IRON
MOTILITY		
FLAGELLA		
Flagellar basal-body P-ring formation protein FlgA	🟩	🟩
Flagellar basal-body rod modification protein FlgD	🟩	🟩
Flagellar basal-body rod protein FlgB		🟩
Flagellar basal-body rod protein FlgC	🟩	🟩
Flagellar basal-body rod protein FlgF	🟩	
Flagellar basal-body rod protein FlgG	🟩	🟩
Flagellar biosynthesis protein FlgN	🟩	🟩
Flagellar hook protein FlgE	🟩	🟩
Flagellar hook-associated protein FlgK		🟩
Flagellar hook-associated protein FlgL		🟩
Flagellar L-ring protein FlgH	🟩	
Flagellar protein FlgJ	🟩	🟩
Flagellar protein FlgO	🟩	🟩
Flagellar protein FlgP	🟩	🟩
Negative regulator of flagellin synthesis FlgM	🟩	🟩
Flagellar biosynthesis protein FlhA	🟩	
Flagellar regulator FlhF	🟩	🟩
Flagellar biosynthesis protein FliL	🟩	🟩
Flagellar biosynthesis protein FliL	🟩	🟩
Flagellar biosynthesis protein FliS	🟩	🟩
Flagellar hook-associated protein FliD	🟩	
Flagellar hook-basal body complex protein FliE	🟩	🟩
Flagellar motor switch protein FliG	🟩	
Flagellar motor switch protein FliM	🟩	🟩
Flagellar motor switch protein FliN	🟩	🟩
Flagellar M-ring protein FliF	🟩	
Flagellar protein FliJ		🟥
Flagellum-specific ATP synthase FliI		🟥
Flagellar regulatory protein FleQ	🟩	
Flagellar synthesis regulator FleN	🟩	🟩
Flagellar rod protein FlaI	🟩	🟩
Flagellin protein FlaA	🟩	🟩
Flagellin protein FlaB	🟩	🟩
Flagellin protein FlaC	🟩	🟩
Flagellin protein FlaD	🟩	🟩
Flagellin protein FlaD	🟩	🟩
Flagellin protein FlaE	🟩	🟩
Flagellin protein FlaF	🟩	
Flagellin protein FlaG	🟩	🟩

Protein	Col 1	Col 2
Flagellar motor rotation protein MotA	green	green
Flagellar motor rotation protein MotB	green	green
Sodium-type flagellar protein MotY precursor	green	green
Sodium-type polar flagellar protein MotX	green	green
54K polar flagellar sheath protein A	green	green
RNA polymerase sigma factor for flagellar operon	green	green
UDP-N-acetylglucosamine 4,6-dehydratase	red	red

PILLI

Protein	Col 1	Col 2
Flp[1] pilus assembly protein	green	green
Flp pilus assembly protein	green	
Flp pilus assembly protein RcpC/CpaB	red	red
Flp pilus assembly protein TadA		red
Flp pilus assembly protein TadB	red	red
Flp pilus assembly protein TadD		red
Flp pilus assembly protein, secretin CpaC	red	red
MSHA[2] biogenesis protein MshE	red	
MSHA biogenesis protein MshF	red	
MSHA biogenesis protein MshG	red	
MSHA biogenesis protein MshL	red	red
MSHA biogenesis protein MshM	red	
MSHA biogenesis protein MshO	red	
MSHA biogenesis protein MshP	red	
MSHA biogenesis protein MshQ	red	
MSHA pilin protein MshA	red	
MSHA pilin protein MshC	red	
MSHA pilin protein MshD	red	

CHEMOTAXIS

Protein	Col 1	Col 2
Chemotactic transducer-related protein	green	
Chemotaxis protein CheC, inhibitor of MCP[3] methylation	red	
Chemotaxis protein CheV	green	green
Chemotaxis protein methyltransferase CheR	green	green
Chemotaxis regulator, transmits chemoreceptor signals to flagellar motor	green	green
Chemotaxis response, phosphatase CheZ	green	green
Methyl-accepting chemotaxis protein	red	red
Methyl-accepting chemotaxis protein	red	
Methyl-accepting chemotaxis protein	green	green
Methyl-accepting chemotaxis protein	green	green
Methyl-accepting chemotaxis protein	green	green
Methyl-accepting chemotaxis protein	green	green
Methyl-accepting chemotaxis protein	green	
Methyl-accepting chemotaxis protein	green	red
Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)	red	
Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)	red	
Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)	red	
Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)	green	
Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)	green	
Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)		red
Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)		green
Methyl-accepting chemotaxis protein, hemolysin secretion protein HylB	red	

Methylase of chemotaxis methyl-accepting protein	
Signal transduction histidine kinase CheA	

[1] Flp: fimbrial low-molecular-weight protein
[2] MSHA: Mannose-sensitive haemagglutinin
[3] MCP: Methyl-accepting chemotaxis protein

Motility-related genes. One of the most relevant results was the high number of flagella-related genes that were found to be regulated by Fur$_{Vvbt2}$ and/or iron. Genes belonging to different operons were identified, and in all cases both Fur$_{Vvbt2}$ and iron regulated them positively. A total of fifteen genes of *flg* operon showed an altered regulation, containing genes mainly for the flagella basal body and hook proteins, and a chaperone, a peptidoglycan hydrolase and a negative regulator (Kim and Rhee, 2003); two genes of the *flh* operon that codify for flagellar regulatory and biosynthesis proteins (Kim *et al.,* 2012); eleven genes of the *fli* operon encoding biosynthesis proteins, motor switch and hook proteins, chaperons, basal body proteins and a ATP synthase; two genes of the *fle* operon with a regulation function (Baraquet *et al.,* 2012); nine genes of *fla* operon that codifiy for flagellin and rod proteins (Kim *et al.,* 2013b); four genes of the *mot* operon for the flagellar motor rotation system (Doyle *et al.,* 2004); and three genes that did not belong to any established operon, including the polar flagella sheath protein A. Interestingly, *fliJ*E, *fliJ* and *fliH* were repressed by iron, both genes related to the flagellar export system of *Salmonella*, a secretion type III system (Minamino and Macnab, 1999), and a gene for an enzyme that is involved in the biosynthetic pathway of pseudaminic acid, a sialic-acid-like sugar used by *H. pylori* to modify its flagellin (Morrison *et al.*, 2008).

Genes for two different pili were found, type IVb (Flp/Tad) and type IVa (MSHA) all of them mainly repressed by iron. Pilus Flp/Tad has been recently proposed as a virulence factor in *V. vulnificus* after a comparison of whole genomes because the cluster is only present in clinical strains (Gulig *et al.,* 2010) and MSHA pilus has been related to environment survival in *V. cholerae* (Chiavelli *et al.,* 2001)

Interestingly, many chemotaxis-related genes are up-regulated and down-regulated by Fur$_{Vvbt2}$ and/or iron. Most of them were subjected to a negative

regulation, although methyl-accepting chemotaxis proteins were under all the regulation types that both Fur$_{Vvbt2}$ and iron can exert. Genes like *cheC, cheV, cheR, CheZ* and *cheA*, that codify for several functions of chemotaxis process, as well as a regulator, a signal-transductor, a chemoreceptor, and a methylase presented an altered regulation in microarray results (Wolfe *et al.,* 1987).

GLOBAL TRANSCRIPTIONAL REGULATORS

Table 4e. Selected genes under control of Fur$_{Vvbt2}$ and/or iron: Gobal transcriptional regulators.

GENE CATEGORY AND NAME	FUR	IRON
GLOBAL TRANSCRIPTIONAL REGULATORS		
QUORUM SENSING		
DNA-binding regulator, LuxR family	🟥	
LuxO	🟩	🟩
LuxP	🟥	🟥
LuxQ	🟥	🟥
LuxT	🟥	
LuxU	🟩	🟩
LuxZ	🟩	
Transcriptional regulator, LuxR family	🟥	
CYCLIC-ADENOSINE MONOPHOSPHATE (cAMP)		
Cyclic AMP receptor protein	🟥	🟥
Predicted signal-transduction protein containing cAMP-binding domain	🟥	🟥
ToxR/S SYSTEM		
Transcriptional activator ToxR	🟥	🟥
Transcriptional activator ToxS	🟥	
FERRIC UPTAKE REGULATOR		
Ferric uptake regulator protein (Fur$_{Vvbt2}$)	🟩	
PROTEINS CONTAINING GGDEF/EAL DOMAINS		
diguanylate cyclase (GGDEF domain) with PAS/PAC sensor	🟥	🟥
diguanylate cyclase/phosphodiesterase (GGDEF & EAL domains)		🟥
GGDEF and EAL domain proteins	🟥	🟥
GGDEF domain family protein	🟥	🟥
GGDEF domain protein	🟩	
GGDEF domain protein	🟩	
GGDEF domain protein		🟩
GGDEF family protein	🟩	🟩
GGDEF family protein	🟩	🟩
GGDEF family protein	🟩	
GGDEF family protein	🟩	

GGDEF family protein		
GGDEF family protein		
GGDEF family protein		
GGDEF family protein		
Putative diguanylate cyclase (GGDEF)/phosphodiesterase (EAL)		
Putative membrane GGDEF domain involved in signal transduction		
Sensory box/GGDEF family protein		

Genes related with global transcriptional regulators. Finally, genes that codify for global transcriptional regulators related to bacterial metabolism, physiology and virulence were also selected. Between these regulators we identified components of the quorum sensing system, of the cAMP-mediated regulation, the ToxR/S system and multiple genes that contain GGDEF (a bacterial ubiquitous domain whose function is to synthesize cyclic di-GMP, used as an intracellular signalling molecule) and/or EAL (a domain found in diverse bacterial signalling proteins that may function as a diguanylate phosphodiesterase) domains, motifs present in proteins involved in regulation and signaling processes. Also, as previously described in the biotype 1 (Lee *et al.,* 2007a), the Fur_{Vvbt2} protein resulted to be positively regulated by itself.

B. Phenotypic characterization of fur_{Vvbt2} mutant

The microarray data suggested that of iron and/or Fur_{Vvbt2}, that presented a 99% identity in aminoacid sequence with Fur_{Vvbt1}, could control several functions related to virulence in *V. vulnificus* Bt2 SerE. To test it, several experiments were designed and phenotype of the wild type strain (grown or not in iron restricted conditions), the mutant and the complemented strain were analyzed and compared.

1. Motility

Results of this assay confirmed that iron, independently of Fur_{Vvbt2}, controls the motility since the colony size directly depended on the quantity of free-iron in the medium (Table 5). In addition, motility was also dependent on Fur_{Vvbt2}, since the

colony diameter corresponding to the wild-type strain was significantly higher than that of the mutant strain in all media with the exception of MA-D (Figure 3 and Table 5). As expected, no significant differences in motility were observed between the wild-type and the complemented strain. Microscopic observation confirmed these results, since motility of fur_{Vvbt2} mutant was clearly reduced when a fresh preparation was observed in all tested conditions (data not shown).

Table 5. Motility of *V. vulnificus* strains in Motility agar.

	Motility[a]		
	CM9	**CM9-Fe**	**CM9-D**
CECT4999	4.8 ± 0.25	6.8 ± 0.34 **	2.6 ± 0.23**
Δ*fur*	3.6 ± 0.52*	5.8 ± 0.11* **	2.1 ± 0.25**
c*fur*	4 ± 0.5	6.7 ± 0.11**	2.1 ± 0.3**

[a] Motility is expressed as the diameter of halo.

** Significant differences for each strain in iron concentration with respect to CM9 (p<0.05)

* Significant differences in fur_{Vvbt2} mutation with respect to the wild-type strain (p<0.05)
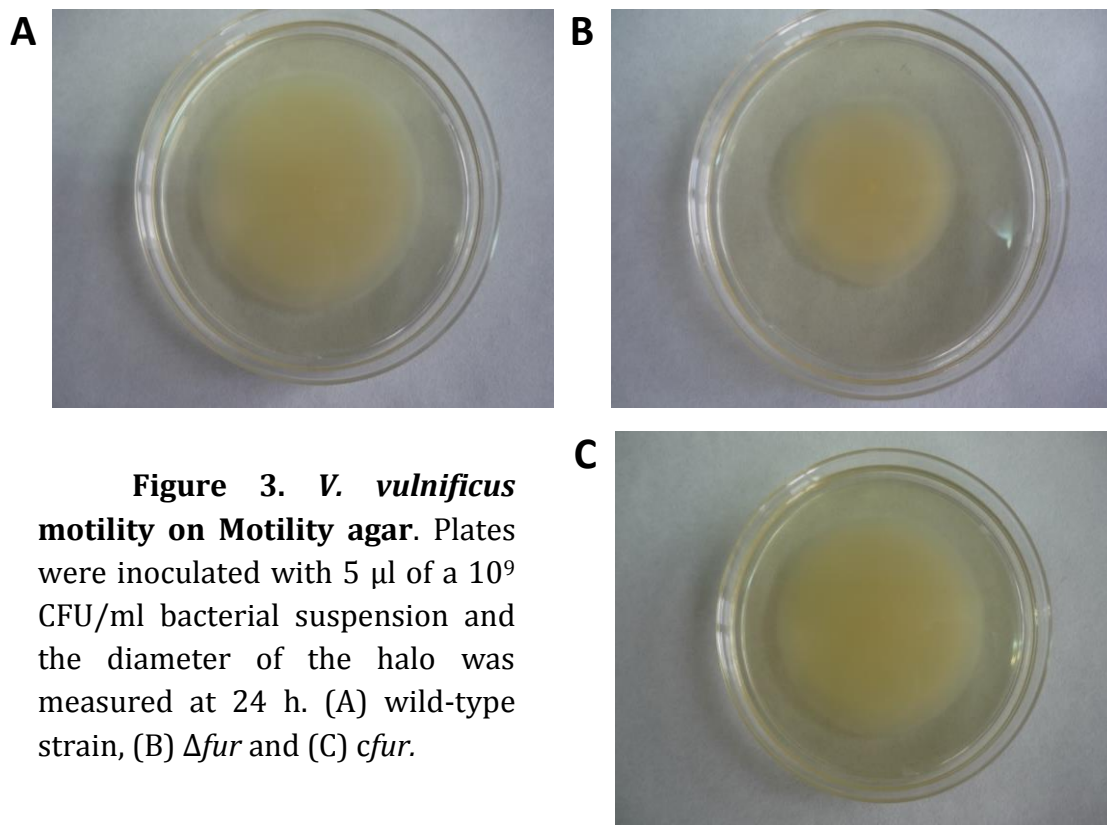
Figure 3. *V. vulnificus* motility on Motility agar. Plates were inoculated with 5 μl of a 10^9 CFU/ml bacterial suspension and the diameter of the halo was measured at 24 h. (A) wild-type strain, (B) Δ*fur* and (C) c*fur.*

2.   Chemotaxis

All tested strains exhibited positive chemotaxis towards eel mucus without significant differences between strains (wild-type *vs* mutant/complemented strain) or conditions (presence *vs* absence of iron) (Figure 4).
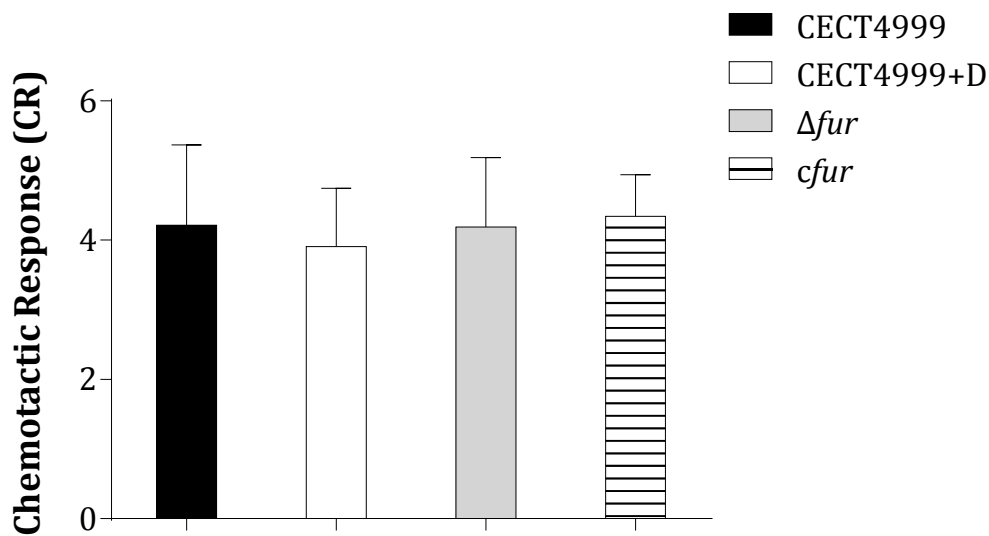
Figure 4. **Chemotactic activity of *V. vulnificus* strains measured as the chemotactic response**.

3.   Attachment to chitin

No DNA was detected at 0 h, indicating that bacteria had not enough time to attach to the chitin surface, while bacteria were detected at 24 and 48 h (Figure 5). Interestingly, the amount of DNA was significantly higher at 48 h than at 24 h (Figure 5). On the other hand, mutation of fur_{Vvbt2} gene or addition of dipyridil to ASW did not affect the bacterial attachment in the assayed conditions (Figure 5).
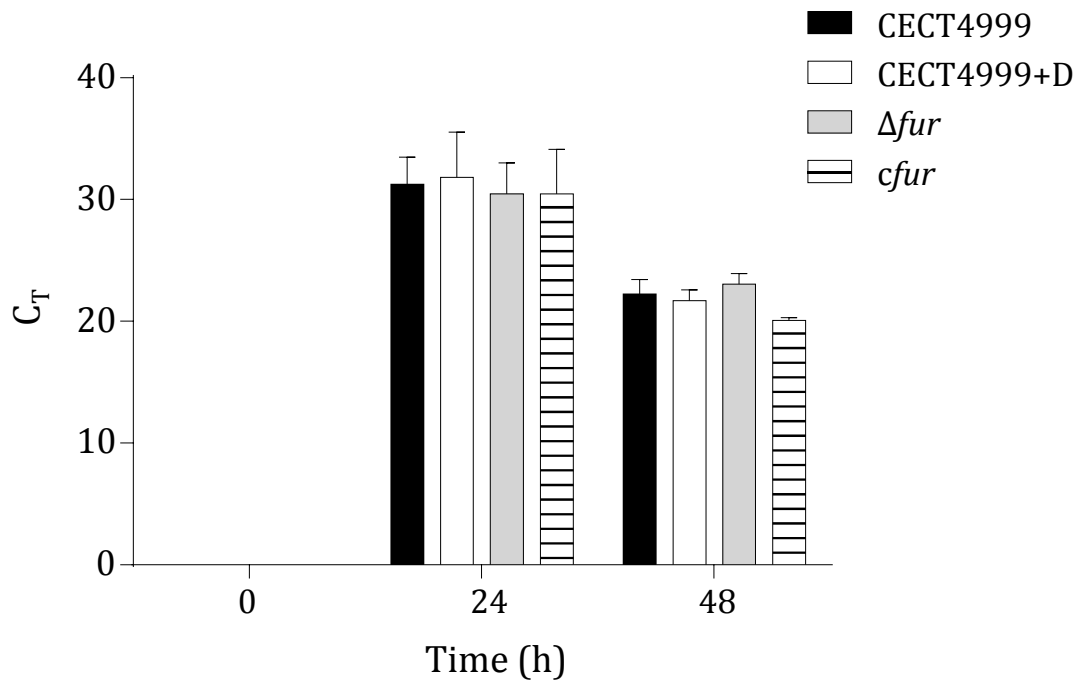
**Figure 5**. **Bacterial attachment to crab chitin measured as quantity of *recA* gene DNA determined by qPCR.**

4. Growth in plasma, hemin and apo-transferrin

The growth of the wild-type and its derivative strains was monitored in artificial media containing apo-transferrin (CM9-Tf) or hemin (CM9-Hm) as the sole iron sources. Although all the strains grew in CM9-Tf and CM9-Hm, the fur_{Vvbt2} mutant entered in the log. growth phase before than the rest of the strains, with significant differences in counts at 7 and 8 h-post incubation in CM9-Hm, and at 7, 8 and 9 h post-incubation in CM9-Tf (Figure 6A and B).
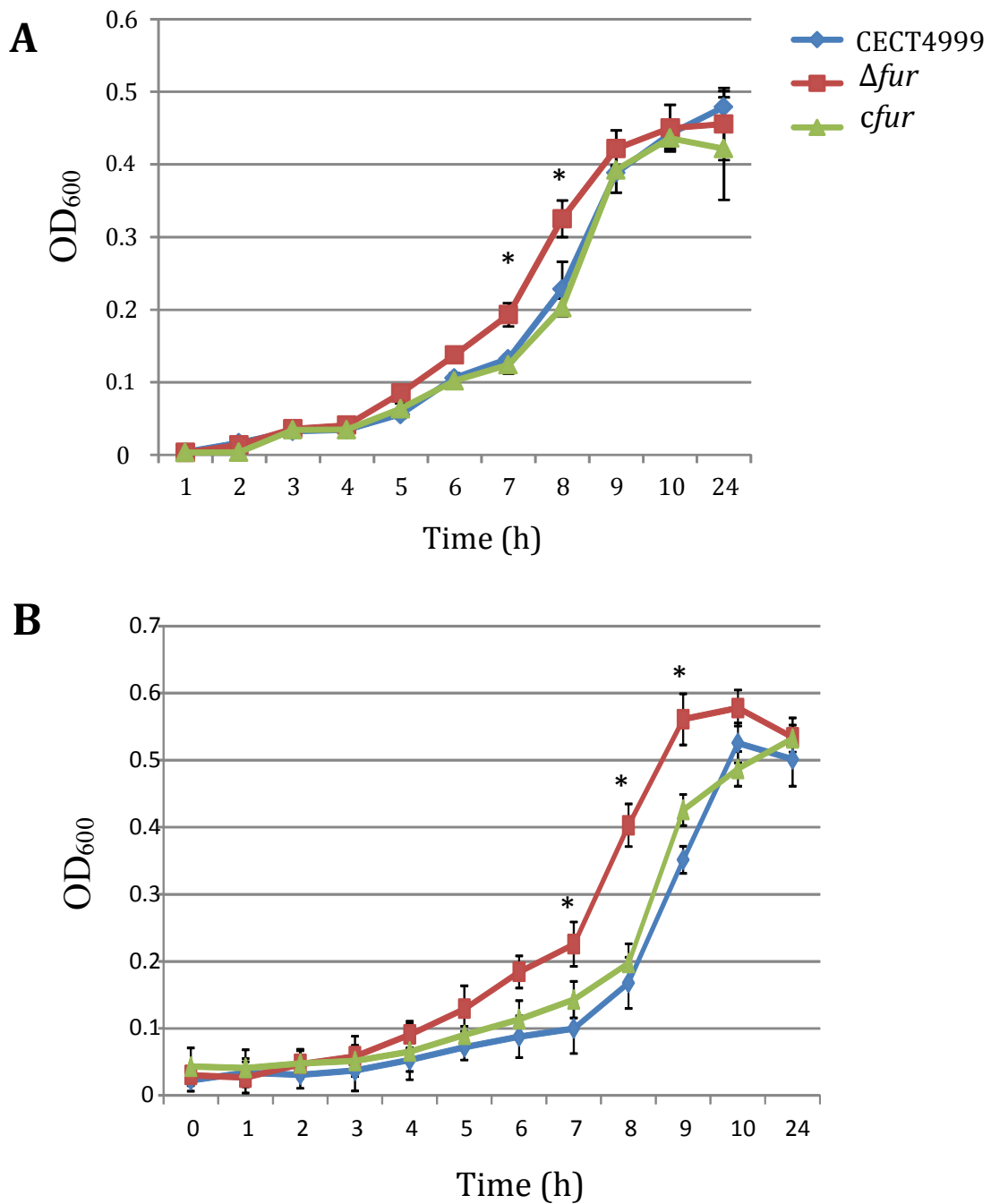
Figure 6. Bacterial growth in presence of hemin and apo-transferrin. *V. vulnificus* and its derivative strains were grown in CM9-Hm (A) and CM9-Tf (B) and growth was monitored in intervals of 1 h by measuring the OD_{600}. Asteriks indicate significant differences in growth between the mutant and the wild-type strain ($p < 0.05$).

Regarding the experiments of growth in plasma, all strains grew in fresh EP (Figure 7) and HP (Figure 8) but grew faster when plasma was inactivated and/or supplemented with iron (Figure 7 and 8). Interestingly, significant differences in growth in favor of Δfur_{Vvbt2} were detected at 4 h and 5 h- post incubation in inactivated-EP and inactivated-HP, respectively (Figure 7 and 8).
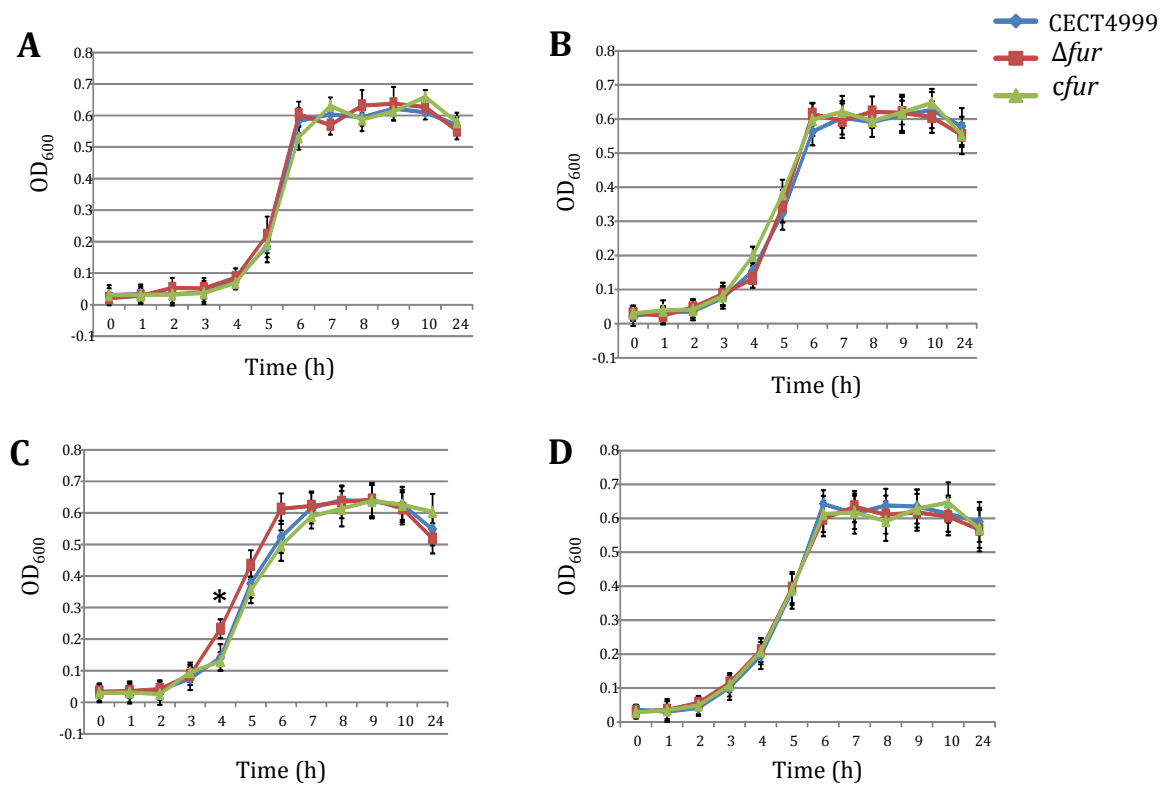
Figure 7. Growth of *V. vulnificus* strains in eel plasma. Growth of wild-type, fur_{Vvbt2} mutant and fur_{Vvbt2} complemented strain in EP (A), EP + FeCl3 (B), heat-inactivated EP (C) and heat-inactivated EP + FeCl3 (D). Asteriks indicate significant differences in growth between the mutant and the wild-type strain (p<0.05).
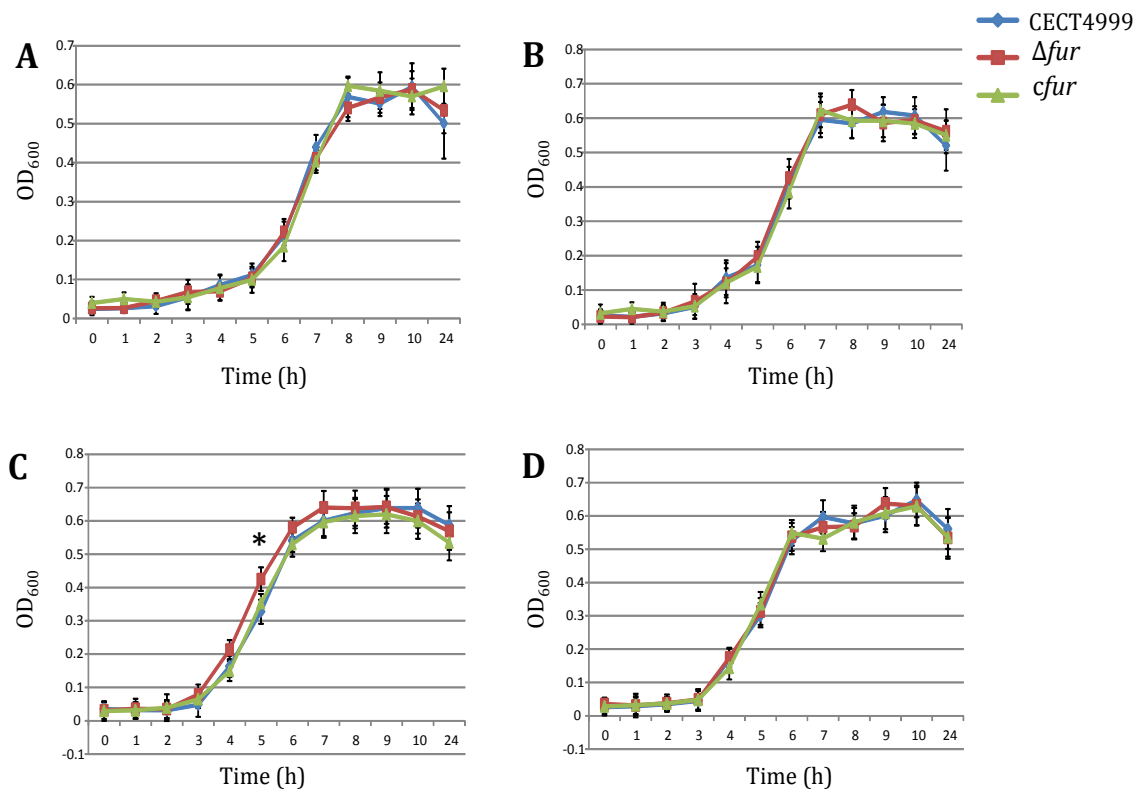
Figure 8. Growth of *V. vulnificus* strains in human plasma. Growth of wild-type, *fur$_{Vvbt2}$* mutant and *fur$_{Vvbt2}$* complemented strain in HP (A), HP + FeCl$_3$ (B), heat-inactivated HP (C) and heat-inactivated HP + FeCl$_3$ (D). Asteriks indicate significant differences in growth between the mutant and the wild-type strain (p<0.05).

5.   MIC's

The three strains showed the same values of MIC for polymixin B, lysozyme, dipyridil and transferrin (Table 6) while the mutant was significantly more sensitive to saponin (a non-ionic surfactant as bile salts) than the wild-type and complemented strains (Figure 9). In this experiment, the mutant population decreased dramatically in the first minutes of incubation in saponin and died after 20 min, while the wild-type and the complemented strains survived.

Table 6. Minimum inhibitory concentration of microcide peptides in growth of *V. vulnificus* strains.

	Minimum inhibitory concentration (MIC)[1]			
	Polimixin B (U/ml)	Lysozyme (µg/ml)	Dipyridil (µM)	Transferrin (µM)
CECT4999	500	500	50	50
Δ*fur*	500	500	50	50
c*fur*	500	500	50	50

[1] MIC was measured as the minimum concentration that inhibited the bacterial growth in CM9 broth.
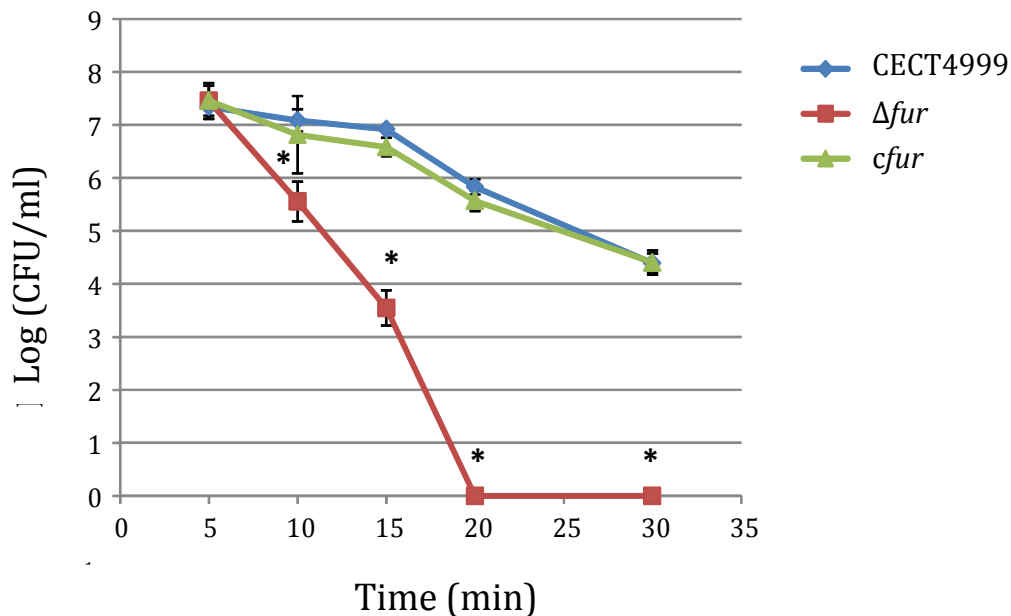
Figure 9. Growth curve of *V. vulnificus* strains in presence of saponin. Bacterial suspensions of each strain of 10^7 CFU/ml were prepared in PBS and saponin was added to a final concentration of 100 µg/ml, and viable bacterial counts were performed by the drop plate method at 5, 10, 15, 20 and 30 min. Asterisks indicate significant differences with respect to the wild-type strain ($p < 0.05$).

6.   Siderophore detection

All the strains produced siderophores of catechol-type without significant differences among them when they were cultured under iron-restriction conditions (Table 7). The mutant strain grown without iron-chelator also produced the same amount of siderophores than wild-type strain under iron-restriction conditions (data not shown).

Table 7. Siderophore production in *V. vulnificus* strains in CM9-Tf.

Strains	Siderophore production[1]		
	Arnow	**Csàky**	**CAS**
CECT4999	+	-	+
Δ*fur*	+	-	+
c*fur*	+	-	+

[1] The criterium for positive or negative result for each test was that of Biosca and cols. (Biosca *et al.*, 1996).

7.   Resistance to shocks

Acid stress. All the strains survived in PBS-1 at physiological pH without differences in bacterial counts over the time (Figure 10A). However, the survival of Δ*fur*$_{Vvbt2}$ at acid pH was significantly higher than that of the wild-type and complemented strains, which showed decreased viability after 120 min (Figure 10B). No differences in survival was detected with regard to the iron content of the medium used to grow the strain (Figure 10).
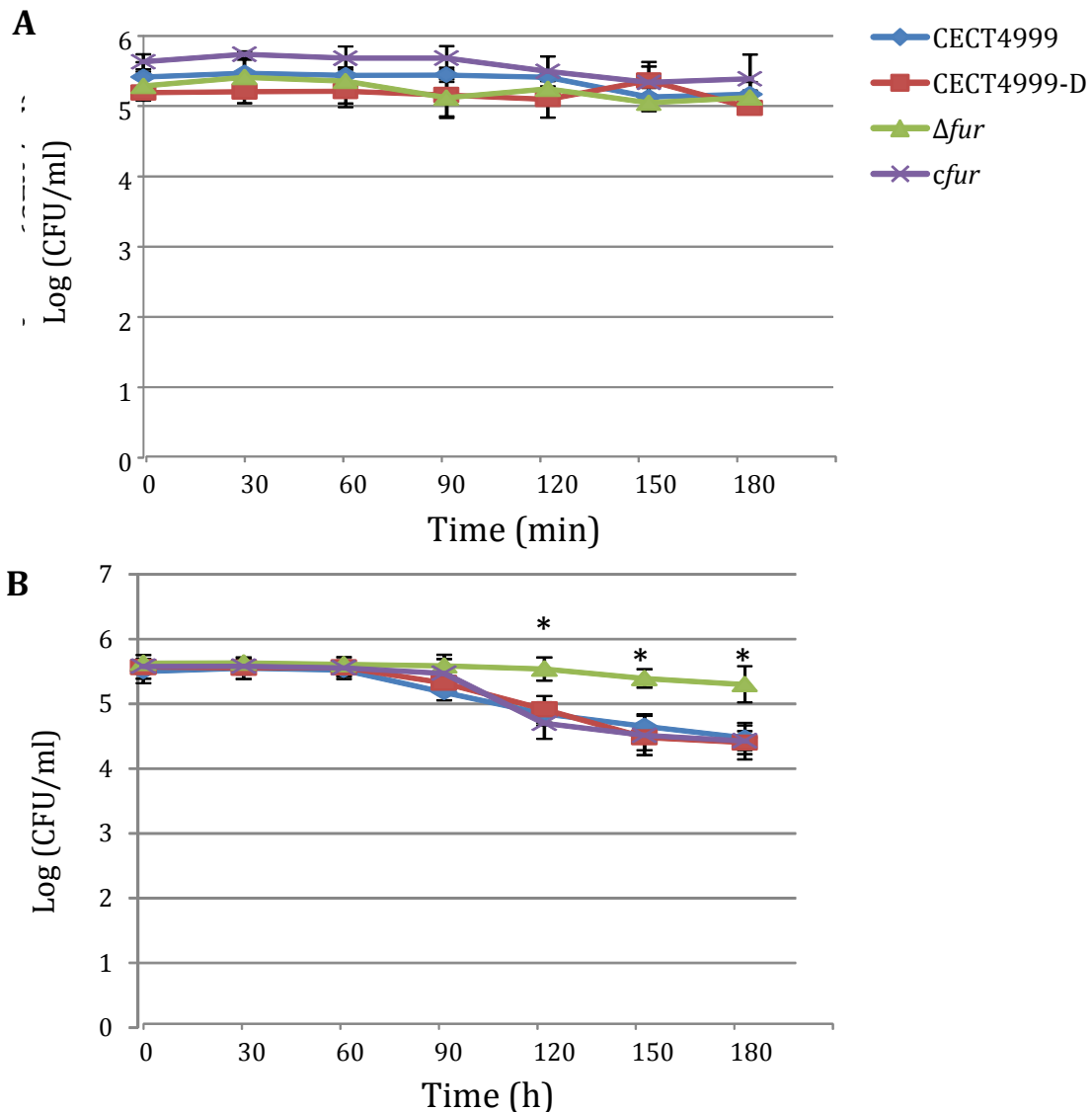
Figure 10. Measurement of acid tolerance of *V. vulnificus*. Bacteria were resuspended in PBS at pH 7 (A) or pH 5 (B) and viable bacteria counts were carried out by the drop plate method. Asterisks indicate significant differences with respect to the wild-type strain (p < 0.05).

Heat-shock. All the strains entered directly in death phase when were incubated at 41ºC without significant differences among them (Figure 11). However, the results also show that pre-acclimatization is a key factor in the resistance to high temperatures (Figure 11); the D value (time required to achieve a survival of 10%) was between 2 and 3 h, for cells pre-acclimated at 28ºC, and around 5 h for the cells pre-acclimated at 37ºC (Figure 11). In all cases, presence of dipyridil did not appear to influence significantly the sensitivity/tolerance to the heat shock.
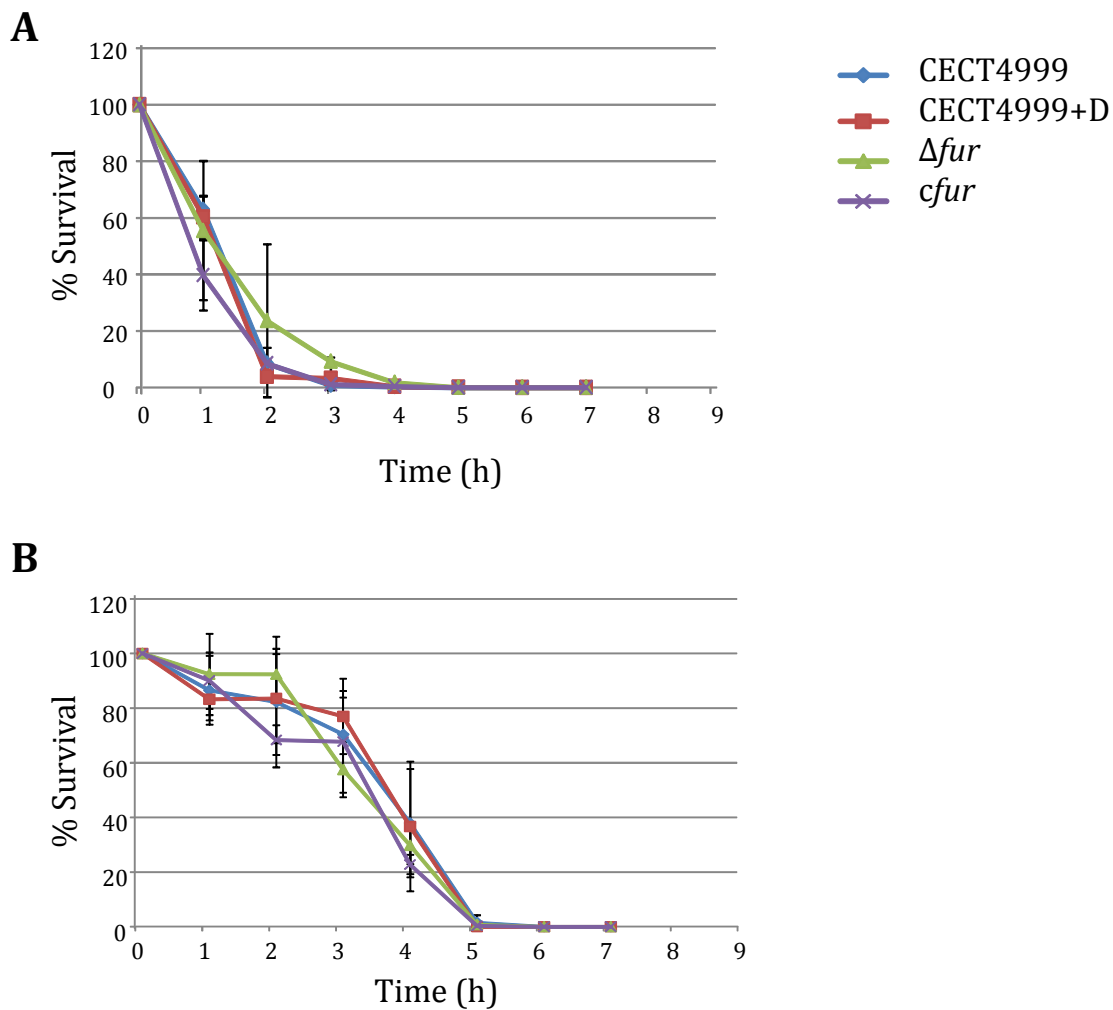
Figure 11. Heat tolerance of *V. vulnificus* strains. Bacteria were grown overnight and incubated in a bath at 41ºC, and viable bacteria counts were carried out by the drop plate method. Bacteria grown in CM9 at 28ºC (A) and in CM9 at 37ºC (B).

Cold shock. In these experiments, the bacteria were maintained for 10 days at 28 and 4 ºC and the survival curves were compared (Figure 12). As expected, bacterial viability decreased gradually and slowly when cultures were held at 28ºC, detecting viable bacteria during more than 10 days (Figure 12A). However, when cultures were held at 4ºC, a faster decrease in number of cultivable bacteria was observed and no cultivable bacteria was obtained at 7 d post-incubation (Figure 12B). In either cases, no difference regarding the strain used or addition of dipyridil were noticed.
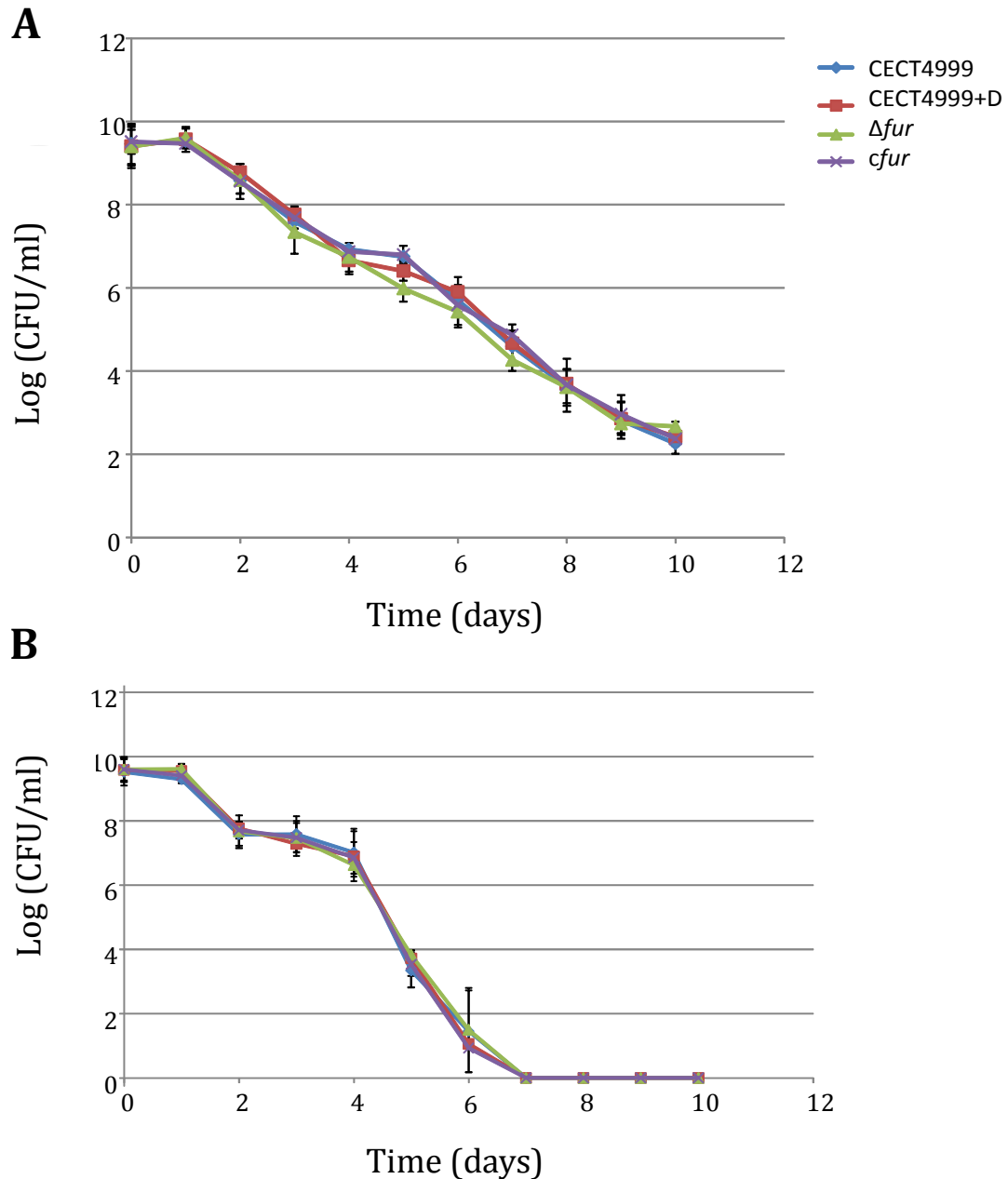
Figure 12. Cold tolerance of *V. vulnificus* strains. Bacteria were grown overnight in CM9 at 28 ºC and maintained during 10 days in 28ºC (A) or 4ºC (B), and viable bacteria counts were carried out by the drop plate method.

8. Proteolytic, hemolytic and chitinase activity

To test if mutation in fur_{Vvbt2} resulted in an alteration of the hemolytic and/or proteolytic, hemolysis of bovine erythrocytes and proteolysis of azocasein by the three strains were assayed. There were no differences among the strains regarding

their hemolytic and proteolytic activity (Figure 13 and Table 8).

Table 8. Proteolytic activity of *V. vulnificus* strains**.**

	Proteolytic activity in proteolytic units (PU)
CECT4999	1094 ± 51.2
CECT4999-D	1151 ± 78.4
Δ*fur*	1131 ± 60.8
c*fur*	1114 ± 112.7
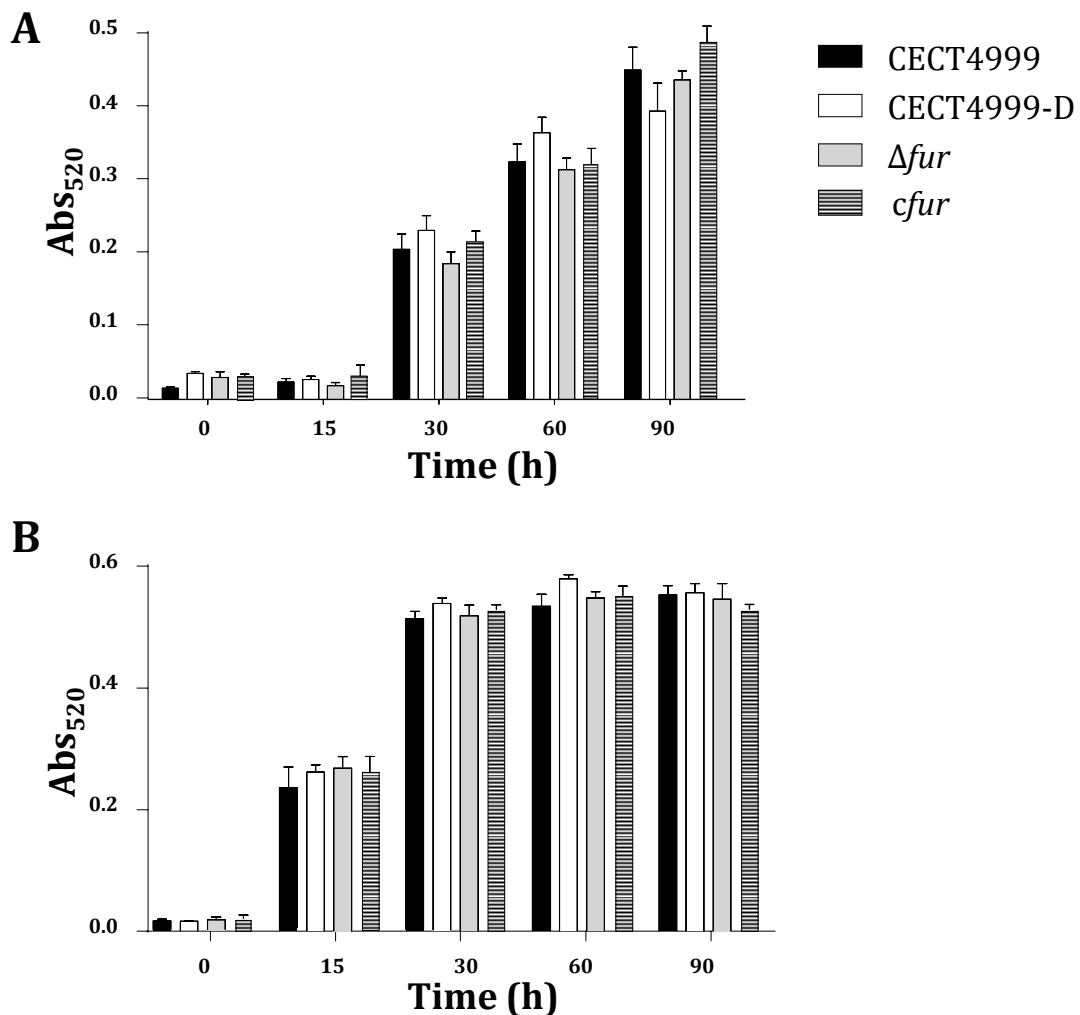
Figure 13. Hemolytic activity of *V. vulnificus* strains. Absorbance of the supernatant of a suspension of 1% bovine erythrocytes incubated with bacterial cells (A) or ECP (B) was measured at 520 nm.

Chitinase activity was quantified as bacterial growth in ASW supplemented with chitin. All strains showed an increase on their growth with a maximum value at 7 days post-inoculation, and a decrease to the levels of day 0 at day 14 post-incubation (Figure 14). No differences were noticed regarding the fur_{Vvbt2} mutation or the medium (with or without dipyridil) (Figure 14).
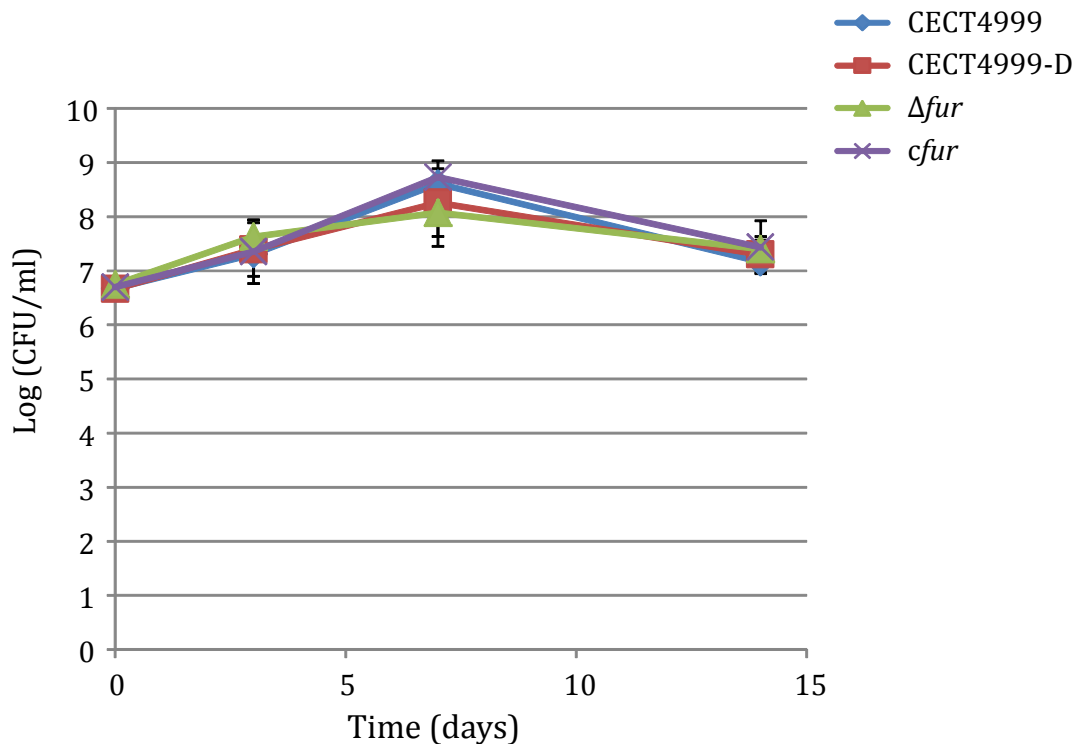
Figure 14. Chitinase activity of *V. vulnificus* strains. Bacteria were grown in ASW plus 1% of colloidal chitin and viable bacteria counts were carried out by the drop plate method

9.   LPS and capsule

The OM extracts from the wild-type, grown in CM9 and CM9-D, and the complemented strain showed the same pattern after immunostaining, which corresponded to a smooth LPS plus the capsule (Figure 15). In contrast, the fur_{Vvbt2} mutant presented a different pattern that affected to the Lipid A mobility, which seemed to have a lower molecular weight.
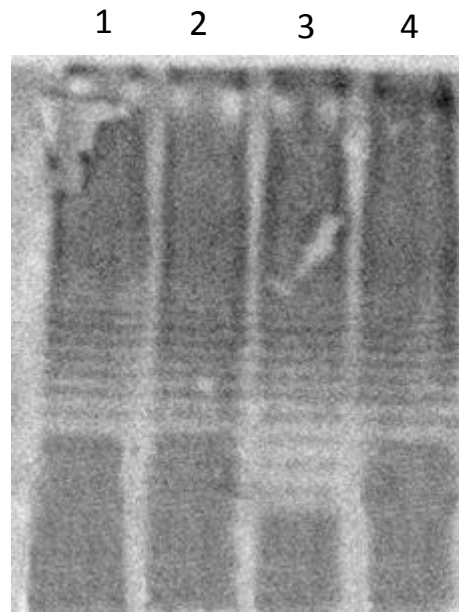
Figure 15. **LPS of *V. vulnificus* stained by Western blot**. LPS was immunostained with rabbit primary antibody anti-CECT4999 and secondary anti-rabbit HRP-conjugated. Lanes contain LPS extracted from wild-type strain grown in CM9 (1), wild-type strain grown in CM9-D (2), Δ *fur_{Vvbt2}* grown in CM9 (3) and c *fur_{Vvbt2}* grown in CM9 (4).

C.   Virulence and colonization/invasion

The *fur_{Vvbt2}* mutant showed an attenuation in virulence degree for eels, independently the route of infection; about 1 log. unit, in the case of i.p. challenge, and about 6-fold in case of bath challenge (Table 9). In both cases, eel death presented a different time death pattern; in the first 2 days post-infection in the case of wild-type strain-infected eels, and in the first 1-5 days, in case of the *fur_{Vvbt2}* mutant strain-infected eels. In all cases, the complemented strain showed the same LD_{50} and death pattern than the wild-type strain (Table 9).

Table 9. Virulence of *V. vulnificus* strains.

Strains	Virulence for eels (LD$_{50}$)[1]	
	i.p.	bath
CECT4999	2.1×10^2	1.2×10^6
Δ*fur*	3×10^3	7.2×10^6
c*fur*	2.5×10^2	2.1×10^6

[1]LD$_{50}$ is expressed as CFU per fish in case of i.p. injection and CFU per ml in case of bath infection of eels (Amaro *et al.,* 1995).

Colonization and invasion experiments revealed that all the strains were able to colonize gills immediately, and latter to spread to the bloodstream and invade all internal organs (Figure 16). Interestingly, *fur$_{Vvbt2}$* mutant was recovered in numbers significantly lower than those of the wild-type and complemented strains at 9 (blood) and 24 h (liver and head kidney) post-infection. In the rest of organs and times, no significant differences were noticed (Figure 16).
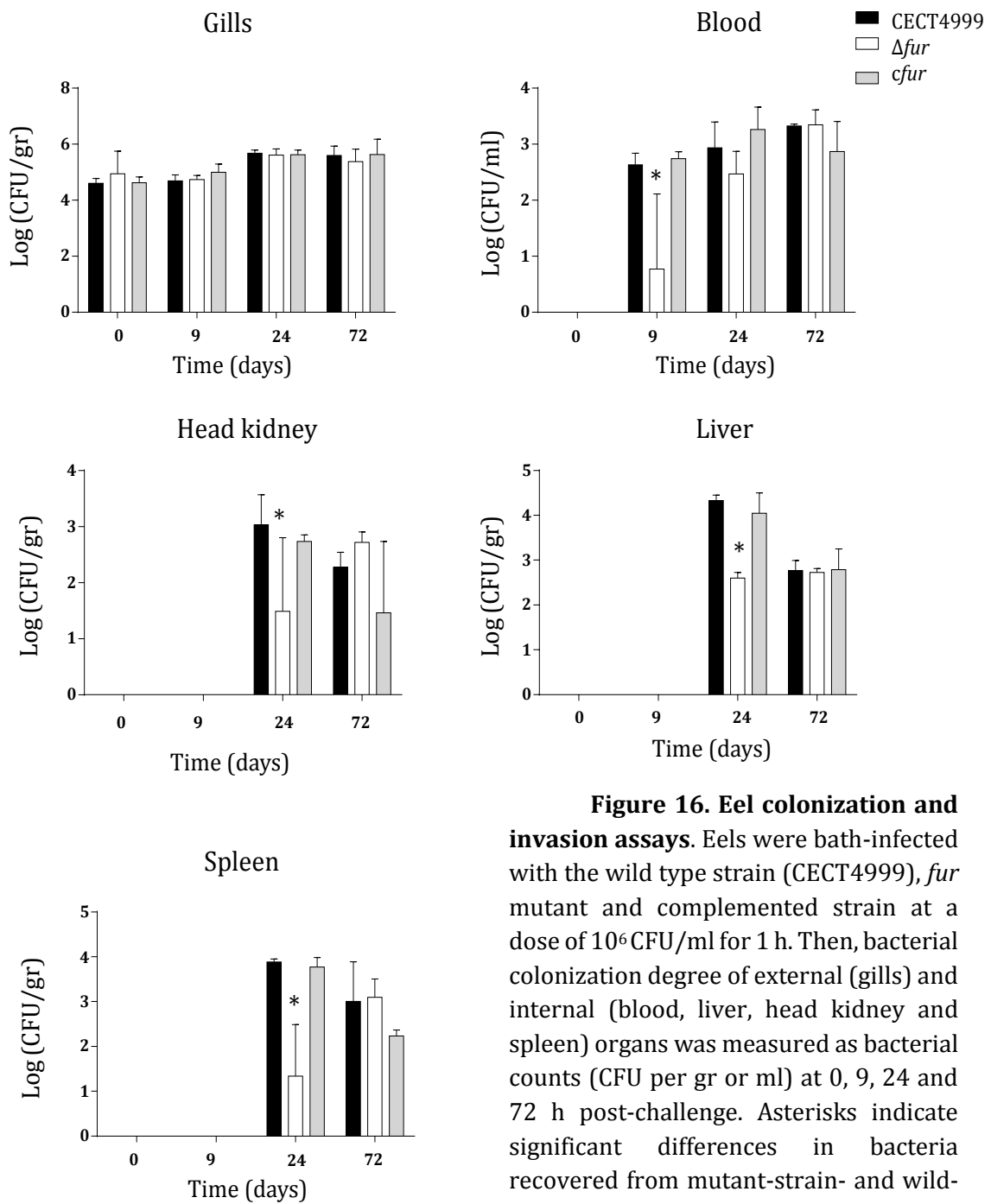
Figure 16. Eel colonization and invasion assays. Eels were bath-infected with the wild type strain (CECT4999), *fur* mutant and complemented strain at a dose of 10^6 CFU/ml for 1 h. Then, bacterial colonization degree of external (gills) and internal (blood, liver, head kidney and spleen) organs was measured as bacterial counts (CFU per gr or ml) at 0, 9, 24 and 72 h post-challenge. Asterisks indicate significant differences in bacteria recovered from mutant-strain- and wild-type- strain-infected eels (p<0.05).

IV. DISCUSSION

Global approaches like DNA sequencing and transcriptome analysis have led to the identification of novel genes involved in host–bacterial interactions as well as to decipher new transcriptional networks. In *V. vulnificus* research, the genome sequencing together with its further analysis by microarray constitutes an essential step forward, as it allows to identify new candidate genes for host–pathogen interaction. The genome of the zoonotic strain used in this work, CECT4999, was sequenced and annotated in 2013 (Prakash *et al.,* 2013, poster communication). This strain is able to infect fish and mice (animal model to test human virulence) and cause death by sepsis. Part of the information required to cause sepsis in fish resides on a virulence plasmid that encodes resistance to fish innate immunity by a mechanism only partially understood. In contrast, the genes required to infect and develop vibriosis in humans are all chromosomal.

The hypothesis of this work was that iron level in host tissues is the common signal to fish and mammals that triggers the expression of virulence factors in the zoonotic serovar of *V. vulnificus*. To test this hypothesis the first step was to obtain a *fur*$_{VvBt2}$ mutant strain since Fur is the main regulator that responds to iron in bacterial pathogens. Fur$_{VvBt1}$ has been proposed to act as a negative regulator that represses the transcription of *fur*-regulon genes in presence of iron. We compared the sequence of Fur$_{Vvbt2}$ with that of Fur$_{VvBt1}$ and found a similarity of 99% in aminoacid sequence, which means that both proteins are homologous. From now, we will refer to this protein as Fur$_{Vv}$. We obtained the mutant in *fur*$_{Vv}$ in our selected zoonotic strain and tested it for virulence. For these experiments, we selected the eel as the animal model on the basis of fishes are good animal models to disentangle the importance of Fur and iron in virulence (Troxell and Hassan, 2013). We infected eels by i.p. injection and by immersion and found that inactivation of *fur*$_{Vv}$ attenuated virulence by both routes in 1 log. unit. Interestingly, the colonization and invasion of internal organs after bath infection was delayed, which suggests that this deficient colonization and invasion of the internal medium was the reason for the attenuation in virulence.

To understand the reason for the decrease in colonization and invasion capabilities exhibited by the fur_{Vv} mutant, our next step was to identify the whole iron and Fur_{Vv} regulons in *V. vulnificus*. To this end, we designed a microarray containing oligoprobes for all the ORFs identified in the genome of our strain. The microarray was validated and used in an experiment performed with the wild-type and the fur_{Vv} mutant strains, grown both in iron rich and iron deficient media.

We found very interesting results. The first one was the high number of genes putatively regulated by iron/Fur_{Vv}, around 1 out of 2, in comparison with the number found in other similar microarray based studies performed in *Vibrio* (Mey *et al.,* 2005; Alice *et al.,* 2008). These genes were equally distributed in ChrI, ChrII and the pVvbt2. Regarding the virulence plasmid, most genes were repressed by Fur_{Vv} and iron. The putative iron/Fur-regulated genes in the plasmid are especially interesting given that they are only present in biotype 2 strains and that elimination of this plasmid abolishes completely the virulence for eels, concomitantly with survival in eel blood (Lee *et al.,* 2008). Among them, we identified, the genes for a transferrin receptor, a putative complement resistant lipoprotein, an anthranilate synthase (required for synthesis of siderophore precursors) and a series of hypothetical proteins, suggesting that the specific virulence for fish attributable to this plasmid is strongly regulated by Fur_{Vv}/iron and that bacteria senses the stimuli of iron concentration in fish hosts. This activation in iron-restriction would ensure that resistance mechanisms to the eel immunity, encoded in the plasmid, will be expressed in blood during the infectious process. The second interesting result was that Fur_{Vv} protein seems to be a very versatile regulator since acts as a repressor and an activator both in presence or absence of iron, which would explain, in part, the high quantity of genes with altered expression found in the microarray experiments. Until now, and except the positive regulation that Fur_{Vv} protein exert on fur_{Vv} gene (Lee *et al.,* 2007a), only a negative regulation of Fur_{Vv} in presence of iron had been described in *V. vulnificus.* However, Fur_{Vc} also acts as a negative and positive regulator, even in conditions of iron depletion, which is in agreement with our results (Mey *et al.,* 2005). There have been described several mechanisms by which Fur can act as an activator. *In vivo* evidence supports the "antirrepressor" activation model as a major mechanism for Fur-dependent activation. In this model Fur

indirectly would repress a gene for a repressor, which in turn would produce an "activation" of the "target" gene (Troxell and Hassan, 2013). Conversely, the repression of several genes could be indirectly conducted by Fur through its interaction with other regulators. We will come back to this item when we analyze the regulators controlled by Fur$_{Vv}$.

Our next step was to design different experiments to test if the differential gene expression showed by the microarray corresponded to a different phenotype that could be related with virulence. To this end, the genes with altered expression were classified in different categories related not only with the infection process but also with survival outside the host, in the environment. In fact, bacteria sense their environment and alter the expression of genes that promote survival both outside and inside their hosts.

V. vulnificus is an aquatic bacterium that can survive as planktonic form or associated to different surfaces mainly containing mucin (i.e. mucous surfaces of fish) or chitin (crabs and zooplankton) (Oliver, 2006). Our microarray results highlight that one chitin binding protein and a MSHA pilus, with a putative role in adherence to zooplankton in *V. cholerae* (Chiavelli *et al.,* 2001), were repressed by Fur$_{Vv}$ and iron, suggesting that bacterial attachment to crabs or zooplankton could be improved in the seawater, an iron-poor environment (Wells *et al.,* 1994). On the contrary, two chitinases were activated by Fur$_{Vv}$ and iron. An experiment was designed to test if attachment to and degradation of chitin were iron/Fur$_{Vv}$ depending processes. We demonstrated that the zoonotic serovar of *V. vulnificus* can grow in the lab by using chitin as the sole carbon source but we did not find differences in growth or attachment between strains or growth conditions. Nevertheless, the experimental design should be improved before to discard the role of Fur$_{Vv}$/iron in metabolism and adhesion to chitin.

Microarray results also revealed that a high number of genes involved in motility, such as those codifying for flagella (including genes for flagellum biosynthesis, flagellins, motor flagellar, etc…) and chemotaxis were up-regulated by Fur$_{Vv}$ and iron. *V. vulnificus* can colonize the mucus because is attracted by mucin, being the flagellum essential for this colonization process. Up-regulating the genes needed for motility, this bacterium ensures a successful colonization of fish mucous

surfaces. We corroborated this positive regulation by the results obtained in the experiments performed in the lab with the wild-type and the mutant strains grown in iron-rich and iron-deficient conditions. In addition, we also found other chemotaxis-related genes regulated by Fur$_{Vv}$ and iron. To test if chemotaxis to mucus was really controlled by Fur$_{Vv}$ and iron, we performed a series of chemotactic experiments. No differences were noticed in chemotactic response towards eel mucus indicating that either Fur$_{Vv}$ and iron do not regulate chemotaxis towards mucus or the phenotype is balanced given that a series of genes were positively regulated and another negatively.

The survival of *V. vulnificus* in starvation in seawater is mainly controlled by temperature. In fact, *V. vulnificus* enters in the VBNC (Viable But Non Culturable) state when temperatures are below 15ºC (Wolf and Oliver, 1992). Our microarray results suggest that genes related to cold resistance would be repressed by Fur$_{Vv}$ and iron and consequently induced in the winter months in seawater, when temperatures are around 4-15ºC. The survival experiments performed at 4ºC revealed that the kinetics of entry into the VBNC state did not vary significantly between strains or conditions. Our next step before to discard the role of iron and Fur$_{Vv}$ in survival in winter will be to analyze the kinetic of resuscitation from the VBNC from the wild-type and the mutant strain grown in iron-rich and iron-poor conditions.

Many genes involved in different iron acquisition systems were identified as repressed by Fur$_{Vv}$ and iron, which means that all these genes would be expressed both in seawater and blood from humans and eels. Among the genes, we identified those of heme transport and vulnibactin biosynthesis and transport, both systems involved in virulence for eels and mammals and expressed *in vivo* and *ex vivo* in iron-deficiency (including fresh plasma and blood) (see Chapter 2) together with the eel-specific iron acquisition receptor *vep20* and genes for uptake and transport of exogenous siderophores such as aerobactin and an uncharacterized siderophore (identified as vibriobactin/enterobactin). This versatility in iron-acquisition mechanisms could be advantageous for this bacterium in the environment where it competes with other bacteria for iron. In addition, the components of a ferrous ion transport system (*feoA, feoB* and *feoC*), genes uncharacterized in *V. vulnificus* but

recently studied in *V. cholerae* (Weaver *et al.,* 2013)*,* were also found to be repressed by Fur$_{Vv}$ and iron. The experiments performed *in vitro* corroborated that iron-acquisition mechanisms by these systems are, in fact, under control by Fur$_{Vv}$ and iron; i.e. the mutant entered in the log phase just before the rest of the strains when were grown in iron-deficient media, suggesting that the iron acquisition systems were constitutively expressed in the mutant.

Apart from genes related to iron acquisition, we also found genes related to survival inside host that were repressed by Fur$_{Vv}$/iron, and, in consequence, of predicted expression in blood and internal organs of fish and humans. One of them was host-specific, the plasmid gene *vep07,* and the other ones host-unspecific. Among the last ones it should be highlighted a series of genes for biosynthesis and transport of LPS, an OM component essential to resist the bactericidal action of serum (Skurnik and Bengoechea, 2009). This is the first time that a putative iron-dependent regulation of LPS biosynthesis in *Vibrio* is reported. In addition, a locus for a Flp pilus was also found as putatively repressed by iron and Fur$_{Vv}$. These kind of pili have not been studied in *Vibrio* but are related to host colonization and adherence to eukaryotic cells in other gramnegative pathogens. (Tomich *et al.,* 2007). On the contrary, Fur$_{Vv}$ and iron induced the transcription of genes related with the resistance to high temperatures, which could be useful when *V. vulnificus* is infecting an iron-overloaded human. This finding is quite interesting because fever is one of the first host defense mechanisms, particularly in those hosts that present an increase in iron concentration in fluids/tissues. In fact, iron-overloading seems to be the most dangerous risk factor because is related with death by sepsis in more than 50% of the clinical cases (Haq *et al.,* 2005). Related to this, we found other genes that could be related to higher resistance to human complement and phagocytosis in iron overloaded humans, such as i) *ktrA* , encoding a K$^+$ efflux pump related to human serum resistance (Chen *et al.,* 2004b), ii) the entire operon for biosynthesis of polysaccharide capsular, related to resistance to human serum and phagocytosis (Wright *et al.,* 1990), iii) genes related to resistance to nitrosative stress, and oxidative stress, needed to resist phagocytosis and, finally iv) genes for resistance to microcidal peptides and to tellurite, a bile salt present in the intestine. Again, it is the first time that a relationship of capsule biosynthesis with iron levels

is reported in *Vibrio*. Regarding stress resistance, microarray results also highlighted other genes involved in this phenotype but repressed by Fur$_{Vv}$ and iron, suggesting that this bacterium could use a different set of genes to resist these innate immunity mechanisms depending on iron availability. Among these genes it could be highlighted *sodA*, a gene encoding the Mg-dependent super oxide dismutase (Mg-SOD) that contributes to overcome the stress caused by acid environment inside the phagocytes, as it has been demonstrated in *V. vulnificus* Bt1 (Kim *et al.,* 2005). Part of these data were confirmed by the experiments performed *in vitro* such as those of growth in eel and human serum, inactivated or not, susceptibility to the bile-salt homologous, saponin, resistance to acid pH, etc... Interestingly, we confirmed that LPS is regulated by Fur$_{Vv}$ since we found that the LPS pattern exhibited by the wild-type and the mutant strains were different, variation that could be also extrapolated to the decrease in virulence degree.

Finally, we found a series of master regulators that seem to be regulated by Fur$_{Vv}$. One of the regulators is LuxR (or SmcR in *V. vulnificus*), a master regulator for the quorum-sensing pathway that collaborates to the orchestration of the expression of virulence genes in *V. vulnificus*, including capsule, biofilm formation and motility (Lee *et al.,* 2007b). We found that Fur$_{Vv}$ and iron repress *luxR* together with a series of genes involved in QS (AI-2 synthetase, sensor...). Previous studies using DNA microarrays to screen for iron-regulated genes in either *V. vulnificus* or *V. cholerae* did not identify this relationship (Mey *et al.,* 2005; Alice *et al.,* 2008). Very recently, Kim and cols. (Kim *et al.,* 2013a) reported the link between Fur$_{Vv}$ and SmcR and concluded that the key is the cell growth phase from which RNA is obtained stationary *vs* logarithmic (the one used in previous works). However, we isolated the RNA from mid-log grown phase cells, which demonstrates that the key is the growth medium but not the growth phase. We used a minimal medium and a minimal medium supplemented with a biological iron chelator, transferrin, both simulating the growth in a host better than complex growth media and chemical iron chelators. The same authors demonstrated that *smcR* is effectively regulated by Fur$_{Vv}$ (Kim *et al.,* 2013a). Related to this, we also found to be apparently regulated by Fur$_{Vv}$/iron a series of genes for biosynthesis and sens to the secondary messanger nucleotide c-di GMP that activates the production of surface adhesins and biofilm

formation and inhibits motility by binding to and regulating transcription factors and or riboswitches.

The high number of genes with an altered expression could also be explained by the fact that the cAMP receptor protein, CRP, was also regulated, and concretely repressed, by Fur$_{Vv}$ and iron. CRP is involved in both metabolism and virulence processes, controlling VvhA, VvpE, the TonB3 system and phenotypes such as capsule production, motility and adhesion (Choi *et al.,* 2002; Kim and Shin, 2010; Alice and Crosa, 2012; Kim *et al.,* 2013c), demonstrating the role of Fur$_{Vv}$ as an indirect regulator in many phenotypic traits. Another master regulator whose expression was repressed by Fur$_{Vv}$ and iron was ToxR. ToxRS genes are found in all *Vibrio* species and form part of the ancestral *Vibrio* genome and control the survival of *V. cholerae* in the environment together with the expression of genes involved in virulence, including the TC pilus (Provenzano and Klose, 2000). One of the most interesting genes controlled by ToxRS, *ompU*, was found to be activated by Fur$_{Vv}$ and iron in this study. OmpU is an OMP that protects *V. cholerae* from bile salts and whose transcription is stimulated by ToxR in the intestine (Provenzano and Klose, 2000). According to this model, the apparent up-regulation by Fur$_{Vv}$ could be indirect through the repression of ToxR. Then, in the iron poor environment that constitutes the intestinal mucus, *toxR* could be transcribed and *ompU* be activated, thus protecting *V. vulnificus* from bile salts. This interesting hypothesis illustrates the cross-talk among regulators and how the apparent activation exceeded by Fur$_{Vv}$ could be indirect through repression of repressors such as LuxR and ToxR. In the same way, some of the genes apparently activated by Fur$_{Vv}$ and iron could be, in fact, under direct control of the QS master regulator and or ToxRS system.

In conclusion, *V. vulnificus* like other pathogenic bacteria, is equipped with complicated signal transduction systems to sense a series of environmental factors that act as "micro-niche markers". Each signal transduction pathway for a single environmental factor, like Fur$_{Vv}$ for iron, has to be inter-related with other signal-pathways, like QS, CRP and ToxRS, to allow the pathogen to response rapidly with the expression of the adequate set of virulence/survival genes. We postulate that Fur$_{Vv}$ is the master regulator that directly or indirectly coordinates the expression

of survival and virulence genes allowing the bacterium to survive in the host and between hosts in the environment.

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GENERAL

DISCUSSION

To understand the pathogenesis of a bacterial species it is essential to characterize its virulence factors and how the immune system responds against them during the infectious process. *V. vulnificus* is a zoonotic pathogen able to cause disease in both mammals and fish, which makes it a very interesting study object. Our aim in the present work was to investigate the role of selected genes in the virulence for mammals and fish as well as determine the genes that form the regulon Fur_{Vvbt2} and those that responds to iron independently of Fur_{Vvbt2}. Additionally, we were interested in separate those host-specific genes from those that are host-specific.

To this end, our strategy was to generate *in frame* deletion mutants in the selected genes and compare their phenotype with the wild-type strain in a wide variety of *in vitro* and *in vivo* assays, to relate gene with function in the infectious process. In case of the regulon determination, the strategy was to use a microarray platform specific for the genome of the strain selected for the whole study, the CECT4999. This platform could be used in further works to analyze the global transcriptomic response to any environmental cues, concretely to those that emulate *in vivo* events to finally understand the relationship of this pathogen with their different hosts.

At the beginning of this thesis, the genome of strain CECT4999 was not available, so we used as a first approach the technic Fur titration assay (FURTA) to identify the genes involved in iron acquisition in this zoonotic serovar. As expected, the FURTA results confirmed that this serovar possesses homologous for the Bt1-genes involved in iron-uptake from vulnibactin (biosynthesis and transport) and heme from host together with genes to use exogenous aerobactin. Surprisingly a plasmid gene for a putative new system for iron-uptake from fish proteins (transferrin?) was also detected.

So, with the aim of understand how iron acquisition systems were involved in the development of the vibriosis in both fish and mammals, we tested single and multiple mutants in *vuuA, hupA hutR* and *vep20* genes (codifying for vulnibactin, heme –two- and transferrin outer membane receptors, respectively) in a series of assays, demonstrating that all the tested genes were induced under iron-restricted

conditions both in artificial growth media and fresh human or eel plasma and that *vuuA*, *hupA* and *vep20* were induced *in vivo* during the eel infection. In accordance, *vuuA*, *hupA* and *vep20* resulted to be virulence genes although *vuuA* and *hupA* are host non-specific and *vep20* is host-specific. Transferrin receptors have been previously identified in some gram negative pathogenic bacteria such as *Neisseria* but never in *Vibrio*. *hutR* would be a secondary heme receptor that could be used as the main receptor by the strains that lack a functional *hupA.* The global phenotypic results suggest that the three genes are used by the pathogen during the first steps of the vibriosis to grow rapidly in bloodstream and overcoming host defenses up to rise a bacterial number enough high to cause the death of the host.

It is important to highlight that *vuuA*- and *hupA*-dependent systems were host-nonspecific, since in both mammals and fish the mutation of the genes had a significant virulence decrease, although these two hosts are not closely related. The results of the sequence and the phylogenetic analysis of both genes showed a moderate variability at sequence level, higher in *vuuA*, and revealed that both belonged to the core genes of the species *V. vulnificus.*

On the other hand, the *vep20* gene, which codified for the Vep20 outer membrane receptor, resulted to be host-specific since its mutation decreased drastically the virulence for eels and only discretely the virulence for mice. Moreover, double mutant in *vuuA* and *hupA* abolished completely the virulence for mice, but still showed a remaining virulence for eels, indicating that this strain must have another iron acquisition system that was not important to virulence for mice but necessary for virulence for eels. Interestingly, this gene was codified in the virulence plasmid pVvbt2, that confers resistance to the eel (and probably teleost) innate immune system, so it has sense that an iron acquisition system specific for eels was in this plasmid acquired by horizontal transfer, highlighting the importance that HGT have in the adaptation of pathogens to different environmental conditions, including the capacity of infect new hosts.

Thanks to iron acquisition systems *V. vulnificus* is able to spread and invade to the host organs and tissues, but this pathogen possess other virulence factors that cause fatal damages in host's structures and that contribute significantly to the

development of the vibriosis. The most important cytolytic factor involved in lysis and destruction of tissues is the MARTX toxin. There are different forms of MARTX toxin in *V. vulnificus*, and that of biotype 2 is the MARTX type III (encoded by *rtxA1₃*). Our results were unexpected since although the double mutant in both *rtxA1₃* copies (the chromosomic and the plasmidic ones) was less virulent for mice and eels (in this case, completely avirulent), it did not present any defect in colonization and invasion of internal organs of the eel. The MARTX type III showed lytic activity towards a wide variety of eukaryotic cells, such as murine macrophages, human epitelium, cyprinid epitelium, eel epitelium, eel phagocytes and erythrocytes from human, sheep and eel, being involved in the resistance to phagocytosis in murine and eel macrophages. The lytic activity of MARTX type III was also observed when strains were incubated in presence of amoeba, their putative natural predator, and the results demonstrated that this toxin is also involved in the protection from the amoeba predation, what strongly suggest that MARTX type III has an important role in the survival on the natural aquatic environment where *V. vulnificus* inhabits, and that can contribute to the first steps of the fish infection since in most cases these kind of amoeba are found in fish gills.

As observed for iron acquisition systems, the *rtxA1₃* gene was induced in blood of infected eels, but *in vitro* did not present overexpression when subjected to several conditions, like excess or restriction of iron, presence of hemin, etc. Only an induction of *rtxA1₃* gene *in vitro* was observed in presence of eukaryotic cells, concretely eel erythrocytes and phagocytes. This results are in agreement with those obtained in MARTX type I of the biotype 1, and explain why this gene was overexpressed *in vivo*.

In the present thesis we also design a microarray platform specific for the biotype 2 serovar E strain, CECT4999, taking as a reference the unpublished genome of this strain. This platform will be very useful in the future to investigate in depth the biology and pathogenicity of the zoonotic serovar of *V. vulnificus* by analyzing the global transcriptomic response. In our case, we use it to determine the genes that are under control of iron, an essential element in regulation of virulence factors of bacteria, and Fur$_{Vvbt2}$ regulator, the main regulator of transcription in response to

iron concentration that controls iron acquisition systems and other virulence factors. Interestingly a high number of genes were under control of both iron and Fur$_{Vvbt2}$, and most of them were genes directly or indirectly related with virulence processes. Virulence-related phenotypes like motility, iron acquisition, toxins, proteases, hemolysins, chemotaxis, LPS and capsule biosynthesis and resistance to stress conditions such as heat, cold, acid, bili or plasma, were strongly regulated (both induced or repressed) by iron and/or Fur$_{Vvbt2}$, in addition of many transcriptional regulators at the same time involved in other different processes. This demonstrated that Fur$_{Vvbt2}$ and iron play an essential role in the pathogenesis of the zoonotic serovar of *V. vulnificus* since they regulate the main virulence factors, as well as many other genes involved in virulence, fact that is a key requirement for any pathogen and that is needed to the correct development of the disease.

Thus, taking together the results of the present thesis, we concluded that *V. vulnificus* possess a repertory of virulence factors that allows the correct development of vibriosis; in the environment, the MARTX type III might contribute to the survival by protecting *V. vulnificus* from predation of amoeba, and possibly other protists, what may increase the probability to contact with the main fish host for the zoonotic serovar, the eel. Once there, the MARTX type III and the iron acquisition systems act coordinately to colonize and invade the internal organs of the eel and destroy the eukaryotic cells and tissues, being all this process regulated by two important factors, the iron concentration and the Fur$_{Vvbt2}$ protein, that coordinates the expression of iron acquisition systems and other many virulence-related processes to finally allow *V. vulnificus* develop the vibriosis and cause the host death by a toxic shock.

CONCLUSIONS

- **MARTX type III** of the *V. vulnificus* biotype 2 is a **key virulence factor** that determines the virulence for both mammals and fish, independently of the route of administration. However, and contrary to that expected, a strain lacking both copies of MARTX type III did not present a defect in colonization and invasion capacity and did not kill the animals, showing that MARTX type III is a virulence factor needed to provoke the host death but **does not seem to have a key role in colonization and invasion capacity**.

- MARTX type III is involved in the **interaction with a wide range of eukaryotic cells**, including red blood cells, professional phagocytes from human, mice and fish, being highly cytotoxic for all of them. MARTX type III is **also involved in killing amoebae**, a putative natural predator of aquatic bacteria, fact that might promote *V. vulnificus* biotype 2 survival in the **environment**.

- MARTX type III **needs to contact** directly to the target eukaryotic cell to exert its lytic activity. Accordingly, our work suggest that bacteria-to-cell **contact is also needed to the expression of *rtxA1$_3$*** and that this gene is also induced in blood of infected eels *in vivo*.

- The three deeply studied **iron acquisition systems** are **important virulence factors** whose role in virulence is to **promote the *in vivo* bacterial growth** in the host internal tissues/organs by scavenging iron from host proteins to **facilitate the spread to the rest of the body** through the bloodstream, up to rise a high population number.

- Two of this systems, the **heme- and the siderophore (vulnibactin)-dependent** (studied taking as reference the *hupA* and *vuuA* genes, respectively), are **host-nonspecific** iron acquisition systems since their inactivation lead to a decrease in both mammal and fish animal models.

- On the contrary, the uncharacterized **Vep20** outer membrane receptor, putatively involved in acquisition of iron from transferrin, seemed to be a **fish-specific** iron acquisition system, data that is in accordance with the fact that Vep20 is encoded in a virulence plasmid that confers resistance to the teleost innate immune system.

- *vuuA* **and** *hupA* **are part of the** *core* **genes of the** *V. vulnificus* **species** and are subjected to variations, probably due to environmental adaptations, while *vep20* was acquired with the virulence plasmid pVvbt2 by HGT conferring an adaptation to the eel. This gene has not varied since its acquisition, which suggests that this has been very recent and also that the gene is under a strong selective force probably exerted by the host.

- We have developed a **microarray platform specific for the zoonotic serovar of** *V. vulnificus* to analyze in detail the global transcriptomic response to any factor that could influence in genetic regulation.

- A **high number of genes were under control of Fur and iron**, being both induced and repressed. Most of the genes were related with virulence, such as those that codifies for flagella, pili, resistance to different shocks, iron acquisition systems, quorum sensing, chemotaxis, exoenzymes and toxins, capsule and LPS.

- The fur_{Vvbt2} mutant assays revealed that **Fur protein is not essential in the development of eel vibriosis and colonization and invasion** of internal organs, but seems to influence slightly in these traits since a small decrease in eel virulence and a retardation in invasion of internal organs were observed, possibly due to the global de-regulation result of the fur_{Vvbt2} mutation and the misspend of resources from the constant synthesis of de-regulated proteins.