



HAL
open science

Phylogeny and life cycle of the zoonotic pathogen *Vibrio vulnificus*

Carla Hernández-cabanyero, Carmen Amaro

► **To cite this version:**

Carla Hernández-cabanyero, Carmen Amaro. Phylogeny and life cycle of the zoonotic pathogen *Vibrio vulnificus*. *Environmental Microbiology*, 2020, 22, pp.4133 - 4148. 10.1111/1462-2920.15137. hal-04648557

HAL Id: hal-04648557

<https://hal.science/hal-04648557v1>

Submitted on 19 Jul 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Phylogeny and life cycle of the zoonotic pathogen *Vibrio vulnificus*

Carla Hernández-Cabanyero and Carmen Amaro *

ERI-Biotecmed, University of Valencia, Dr. Moliner, 50, Valencia, 46100, Spain.

Summary

Vibrio vulnificus is a zoonotic pathogen able to cause diseases in humans and fish that occasionally result in sepsis and death. Most reviews about this pathogen (including those related to its ecology) are clearly biased towards its role as a human pathogen, emphasizing its relationship with oysters as its main reservoir, the role of the known virulence factors as well as the clinic and the epidemiology of the human disease. This review tries to give to the reader a wider vision of the biology of this pathogen covering aspects related to its phylogeny and evolution and filling the gaps in our understanding of the general strategies that *V. vulnificus* uses to survive outside and inside its two main hosts, the human and the eel, and how its response to specific environmental parameters determines its survival, its death, or the triggering of an infectious process.

Introduction

Vibrio vulnificus is an aquatic bacterium that inhabits marine and estuarine ecosystems located in temperate, tropical and subtropical areas. The species is a zoonotic agent with the ability to infect a wide variety of hosts during its life cycle, from humans to different fish species. This review tries to give to the reader a wide vision of the biology of this pathogen covering aspects related to its phylogeny and evolution and filling the gaps in our understanding of the general strategies that *V. vulnificus* uses to survive outside and inside its two main hosts, the human and the eel.

Taxonomy and phylogeny: A new proposal for intraspecific classification

Vibrio vulnificus was defined as a new bacterial species within *Vibrio* genus by Farmer in 1979. The species included a series of phenotypically unusual vibrios (lactose and indole positive) that had been isolated from clinical (human) and environmental (seawater and bivalve) samples in the United States (Farmer 3rd, 1979). At about the same time, bacteria from the same species but from diseased farmed-eels were isolated in Japan (Muroga et al., 1976). The Japanese strains constituted a new serological group within the species (later designated as serovar E [Bt2-SerE] by Biosca et al., 1996) and differed from the American strains in a few biochemical tests (Tison et al., 1982). Consequently, the species was divided into two biotypes, one to group the original isolates, which were serologically heterogeneous (Bt1), and the other one to cluster the eel isolates (Bt2), which were serologically homogeneous (Tison et al., 1982).

Later, both biotypes were isolated worldwide and, concurrently, the serological variability of Bt2 was reported by various laboratories (Høi et al., 1998; Biosca et al., 1999; Fouz and Amaro, 2002; Fouz et al., 2006). Remarkably, Bt2-SerE was confirmed to be a zoonotic agent since strains of this serovar were identified as the causative agents of several human infections after diseased eel handling (Veenstra et al., 1992; Amaro and Biosca, 1996; Dalsgaard et al., 1996). On the basis of these results, it was concluded that both biotypes had the potential to infect humans but only Bt2 could also behave as a zoonotic agent (Amaro and Biosca, 1996). Later, the reason why Bt2 could infect fish was clarified when it was shown that this ability resided in a virulence plasmid (pVvBt2) that could be transferred among strains by parasitizing a widely distributed conjugative plasmid (Lee et al., 2008; Roig and Amaro, 2009). Subsequently, a third biotype (Bt3) was isolated from outbreaks of severe wound infections and septicemia cases among fish farm workers and consumers in Israel (Bisharat et al., 1999). The Bt3 grouped the strains of the species with a few distinctive phenotypic traits that constituted a serologically and genetically homogeneous group (Bisharat et al., 1999, 2007). This biotype was later isolated from healthy tilapia and tank water but never from diseased tilapia (Broza et al., 2009, 2012; Efimov et al., 2013). To date, Bt3 has only been isolated in Israel.

The subdivision of the species into biotypes has historically been maintained despite the phenotypic variability exhibited by the *V. vulnificus* isolates that have been recovered over the years (Sanjuán et al., 2009). This variability called into question the biological and even practical meaning of this sub-specific classification. To clarify this issue, a series of genotypic studies were performed. All these studies suggested that *V. vulnificus* species was divided into two main clusters that did not correspond to the three biotypes, one dominated by strains from environmental sources (Environmental [E]), and the other one dominated by strains from human clinical samples (Clinical [C]) (Nilsson et al., 2003; Bisharat et al., 2005; Cohen et al., 2007; González-Escalona et al., 2007). Later, Sanjuán et al. (2011) showed that the species was divided into three main clusters (again without correspondence with the

biotypes) after analysing the variability of seven housekeeping and virulence genes by Multi Locus Sequence Analysis (MLSA) in more than 100 strains of the three biotypes isolated from clinical (animal and human) and environmental sources (Sanjuán et al., 2011). These authors also found that Bt2 was polyphyletic with a correspondence between serovar and sub-cluster, while Bt3 was monophyletic and Bt1 highly polyphyletic. A complementary e-Burst analysis from the sequence types detected only two clonal complexes in the species (Bt2-SerE and Bt3), both associated with fish farms environments (unpublished results).

With the advent and popularization of mass sequencing techniques, numerous strains of *V. vulnificus* were sequenced and their genomes were deposited in databases (Park et al., 2011; Li et al., 2012; Morrison et al., 2012; Wang et al., 2012; Danin-Poleg et al., 2013). The analysis of these genomes could clarify the evolution of the species and help to find a new sub-specific classification system with a solid biological basis. Among these works, we can highlight those of Koton et al. (2015), Roig et al. (2018) and López-Pérez et al. (2019). Koton et al. (2015) performed a genomic comparison and found again that the species was divided into two main clusters, one of them including strains of the three biotypes, mostly from the environment (E-cluster), and the other one grouping Bt1 strains mostly from human clinical origin (C-cluster). The authors hypothesized that Bt3 had evolved from environmental populations in the fish-farming environment and formed a genetically distinct group within the E-cluster (Koton et al., 2015). López-Pérez et al. (2019) combined a genomic comparison with a phenotypic analysis in a wider collection of strains and found four different clusters in *V. vulnificus* that shared mobile genetic elements. The authors concluded that the two largest clusters (C1 and C2, equivalent to the clusters C and E respectively) were apparently adapted to different lifestyles, bloomer (opportunistic) and commensal, but both coexisted on the mucosa of aquaculture species. Thus, members of cluster C1 would be better adapted to cope with the rapid and drastic ecological transition under the unfavourable conditions of the oligotrophic aquatic environments while members of C2 would have a competitive advantage for colonization and growth in different hosts following a commensal lifestyle. The authors propose that strains from the two clusters occupy different niches that lead over time to a greater divergence that would likely eventually lead to the speciation of the two clusters. Finally, Roig et al. (2018) performed a deeper phylogenomic study and showed that the species was subdivided into five well-supported phylogenetic lineages. The five lineages share the virulence genes involved in human pathogenicity, which means that all strains of the species could be pathogenic for humans. This study also showed that the virulence plasmid is the only trait that gave coherence to Bt2, a result already suggested by Sanjuán et al. (2011).

The authors also found a lack of congruence between the phylogeny of plasmid and chromosomes, which suggested that the plasmid had been acquired by different clones in independent events. One of these clones was very successful, spreading worldwide and giving rise to the current zoonotic clonal-complex (Roig et al., 2018). Finally, the authors highlighted the role of fish farms as 'genetic hotspots' for gene transfer and suggested that pVvBt2, currently restricted to L2, could be spread to other lineages. Based on these results, Roig et al. (2018) proposed to subdivide the species into lineages and to assign the isolates that possess the virulence plasmid to a pathovar with the ability to infect fish (*pv. piscis*). If this classification were accepted, it would be necessary to design some tests to discriminate between lineages, probably some PCR targeting lineage-specific genetic markers. One of these tests has already been designed and validated. This PCR identifies the strains belonging to L3 with a 100% of probability (MS in preparation).

In conclusion, *V. vulnificus* species is subdivided into at least five phylogenetic lineages and a pathovar able to infect fish. Within this pathovar there is a zoonotic clonal complex that has been very successful and has spread worldwide. Finally, it should be highlighted that all these genomic works coincide in

emphasizing the role of fish farms as evolutionary motors that accelerate genetic exchanges and favour the emergence of new pathogenic variants in the species *V. vulnificus*.

Diseases

V. vulnificus causes in humans and aquatic animals a disease with multiple clinical manifestations collectively known as vibriosis. Curiously, both human and fish vibriosis can be transmitted either by contact or ingestion and in all cases, the most severe form of the disease is an acute septicemia with a high probability of death by sepsis in less than 24–72 h (Jones and Oliver, 2009; Fouz et al., 2010; Amaro et al., 2015; Oliver, 2015). *V. vulnificus* is considered a pathogen of animal and public health concern as it is responsible for multiple outbreaks in fish farms, especially in Europe and Asia, as well as for the majority of seafood-borne human deaths, especially in the United States (Kaspar and Tamplin, 1993; Tao et al., 2012).

Human vibriosis

The disease caused by *V. vulnificus* in humans occurs as sporadic cases of severe wound infections after contact with seawater or mild gastroenteritis following the ingestion of raw shellfish. Zoonosis cases are all severe wound infections after diseased fish handling. All these infections can converge to septicemia (primary in case of ingestion and secondary in case of contact), which can cause death in risk patients (Oliver, 2015). Primary and secondary sepsis present the same clinical signs, making differential diagnosis difficult (Fig. 1) (Oliver, 2015). Remarkably, the only described cases of human vibriosis outbreaks are all associated with fish farmers and fish consumers, stressing the role of fish farms and fish as a common source for human infections. According to Roig et al. (2018), all strains of the species are potentially virulent for humans. However, there is an apparent association between the lineage and the preferred route of infection, which may reflect the habitat to which the bacterium is adapted to live. Thus, strains of L1 (bloomers according to López-Pérez et al. (2019)) would preferentially infect by ingestion, while strains of the other lineages (commensal according to López-Pérez et al. (2019)) would preferentially infect by contact.

Independently of the virulence of the strain and the form of the disease, the progression of the infection to sepsis clearly depends on the infected patient's gender and health status (Jones and Oliver, 2009; Horseman and Surani, 2011; Baker-Austin and Oliver, 2018). Epidemiological data on this pathogen show that around 86% of the reported cases were males, independently of the route of infection. This data point out that males are six times more likely than females to be infected with *V. vulnificus* (Jones and Oliver, 2009; Baker-Austin and Oliver, 2018). In healthy patients, the disease is usually self-limiting and rarely leads to septicemia, while in susceptible patients the disease can lead to death by sepsis depending on underlying diseases (< 5 % cases occur in individuals without any identifiable risk factor) (Horseman and Surani, 2011). The main diseases predisposing to sepsis are chronic liver diseases (cirrhosis, hepatitis, liver transplantation, or liver cancer) and hemochromatosis, a hereditary blood disease (Horseman and Surani, 2011; Menon et al., 2014). Nutritional immunity due to iron sequestering by transferrin seems to be the key defence mechanism to keep *V. vulnificus* under control. Precisely, the two main risk factors (chronic liver diseases and hemochromatosis) can lead to iron-overload in serum because of low transferrin and hepcidin (an iron-regulatory hormone) production and hereditary hepcidin deficiency, respectively. The most complete epidemiological dataset on the human diseases caused by *V. vulnificus* comes from the COVIS system from the United States (https://www.cdc.gov/vibrio/surveillance.html?CDC_AA_refVal=https%3A%2F%2Fwww.

cdc.gov%2Fnationalsurveillance%2Fcholera-vibrio-surveillance.htm). According to COVIS data, there is a tendency in the last years towards an increase in the number of *V. vulnificus* wound infections and stabilization of foodborne infection cases. The increase in wound infections could be related to the increase in water temperature due to global warming while the stabilization of foodborne infections could be due to the preventive measures applied in the most affected states that have counteracted this global tendency (Baker-Austin et al., 2010; Vugia et al., 2013; Baker-Austin and Oliver, 2018). Finally, it is important to be aware that not only global warming but also other factors such as ageing population, an increase of patients with predisposing risk factors (meaning those with high levels of free iron in the blood caused by different underlying pathologies) and more exposure [i.e. wounds exposed to water and changes in dietary habits (more consumption of raw seafood or sushi)] could lead to an increase of *V. vulnificus* infections in the future.

Fish vibriosis

In fish, *V. vulnificus* causes a disease known as warm water vibriosis because it occurs in warm and tropical areas (Amaro et al., 2015). The disease is probably a common disease in fish, and carrier fish act as a reservoir for the pathogen (Esteve et al., 2007; Carda-Diéguez et al., 2017). The main risk factor for fish health is water temperature, as fish are much more susceptible to *V. vulnificus* infection at temperatures above 25°C (Amaro et al., 1995). This increased susceptibility does not appear to be related to a malfunctioning immune system, as these temperatures are in the natural temperature range of the affected species (Sadler, 1979). The disease is mainly transmitted through water and has two modalities that depend on the causal agent. The modality caused by the zoonotic clonal-complex of pv. *piscis* affects eels cultured in brackish water (0.7%–2% NaCl) and appears as high-mortality outbreaks. In this case, the pathogen uses the gills as the main entrance portal into the fish body (Marco-Noales et al., 2001). The modality caused by the other pv. *piscis* serotypes affects eels and tilapia cultured at lower salinities (0.3%–0.9% NaCl) and occurs as recurrent low-mortality outbreaks (Biosca et al., 1991; Amaro and Biosca, 1996; Chen et al., 2006). In this case, the pathogen preferentially colonizes the intestine and anus (Fouz et al., 2006). Although there are some differences in clinical manifestation between infected fish by oral or contact route, the main clinical signs are common and consist of abdominal petechiae, haemorrhages at the base of the dorsal and anal fins and redness in the region of the operculum (Fig. 1) (Amaro et al., 2015).

Life cycle of *Vibrio vulnificus*

Figure 2 summarizes the life cycle of *V. vulnificus*. Given the relevant role of exogenous iron in the severity of human vibriosis as well as the water temperature in the incidence of fish vibriosis, we have considered pertinent to analyse the current knowledge on the role of both parameters in the life cycle of this microorganism and how the response to these environmental parameters could determine the survival inside and outside its main natural hosts, humans and eels.

Survival in water

The pathogen inhabits natural marine and estuarine waters of salinity between 0.2% and 2.5% salt, (optimum around 1%–1.8%) and temperature between 10 and 33°C (optimum around 25–28°C) (Oliver, 2015; Baker Austin and Oliver, 2018). *V. vulnificus* is also a common inhabitant of artificial

brackish water ecosystems (salinity 0.3%–2%) such as fish farms, especially those where water is kept over 20°C (Biosca et al., 1991; Amaro and Biosca, 1996; Chen et al., 2006; Fouz et al., 2006).

V. vulnificus is very sensitive to low temperatures and has developed a strategy to survive below 10°C by entering into a dormant state known as viable but not cultivable (VBNC) in which it can survive for years (Oliver, 2015). The pathogen can also enter into this state as a result of cold-induced hypersensitivity to oxidative stress, a process independent of iron but controlled by Fur (the main regulator of iron uptake) through aphC (subunit C of alkyl hydroperoxide-reductase) (Pajuelo et al., 2016). *V. vulnificus* resuscitates from the VBNC state with an increase in temperature in the presence of nutrients (Wolf and Oliver, 1992; Whitesides et al., 1997; Marco-Noales et al., 1999). Since nutrients are abundant in the habitats of the pathogen associated with human and animal infections (coastal areas and fish farms), temperature is probably the only epidemiologically relevant environmental factor. Consequently, climate change will not only increase the geographical distribution of the pathogen but also the proportion of cells that are metabolically active and infective throughout the year.

Vegetative cells of the species can survive in water, either in a free-swimming form or in a sessile form attached to organic and inorganic surfaces (Oliver, 2015). According to the hypothesis of López-Pérez et al. (2019), bloomers would be more abundant in the water column and commensals would be more abundant on the mucous surfaces, especially those of fish. Consequently, water and filtering organisms would be the main environmental reservoir for bloomers, and fish would be the main reservoir for commensals, which would support the finding of Roig et al. (2018) on the apparent association between lineage and preferred route of infection. It should be noted that strains belonging to the zoonotic clonal-complex (L2), those related to cases of zoonosis from handling diseased fish, have not been isolated from oysters nor are they associated with shellfish-borne human infections (Amaro et al., 2015), which strongly support this hypothesis.

Colonization of the host

Regardless of the ecotype of the strain, motile bacteria can be attracted by the nutrients present on biotic and abiotic surfaces, a process that is enhanced under iron-overload conditions and at temperatures above 25°C (Fig. 2) (Pajuelo et al., 2016; Hernández-Cabanyero et al., 2020). In fact, multiple chemotactic, flagellum and motility genes, as well as a number of related regulators, are controlled directly or indirectly by Fur in an iron dependent manner, as well as by temperature (Table 1).

In addition, the concentration of iron in the environment, but not temperature, also controls the production of capsules and pili (Table 1) (Pajuelo et al., 2016; Hernández Cabanyero et al., 2020). In general terms, Flp (Cpa) and MSHA pili are overexpressed under iron-deficient conditions, while the capsules are overexpressed under iron-stressed conditions (Table 1). These pili play an important role in the early stages of biofilm formation (Paranjpye and Strom, 2005), while capsules interfere with biofilm formation in *V. vulnificus* (Casper-lindley and Yildiz, 2004; Joseph and Wright, 2004). Consequently, the formation of biofilms would be favoured under iron deficient conditions, while bacterial dispersion of biofilms in the water would be favoured in the case of an excess of iron (Fig. 2).

To colonize the host, the bacteria must first be chemo attached to the mucin of the mucosal tissues or the blood released from open wounds. Chemo-attraction depends on the exoprotease *Vvp* (or *VvpE*), as knockout mutants in *vvp* are attracted neither to mucin nor to haemoglobin and are defective in fish colonization *in vivo* (Valiente et al., 2008). *Vvp* is a multifunctional protease that is capable of

degrading multiple proteins *in vitro*, including mucin and haemoglobin (Miyoshi et al., 1997, 1999; Miyoshi, 2006), so it is likely that degrading them *in vivo* will release amino acids that attract more bacteria to the tissue, thus promoting colonization (Miyoshi et al., 1997; Valiente et al., 2008). Although *vvp* transcription does not respond to temperature changes, the exoprotease activity of the bacteria (mostly attributed to Vvp) is negative at 20°C, minimum at 25°C and optimal at 28°C followed by 37°C (Elgaml and Miyoshi, 2017; Hernández-Cabanyero et al., 2020).

Once in the mucosa (superficial or intestinal) or in a wound, the priority for the pathogen is to adhere, otherwise, it will be carried out by water or peristaltic movements of the intestine. In the case of mucosal surfaces, *V. vulnificus* may first adhere to the mucin on its surface (e.g. using the colonization factor GbpA [GlcNAc A-binding protein]) and then, once covered with mucin, it adheres to the different mucin receptors on the epithelial cells (Jang et al., 2016). At this point, Vvp may have another interesting function, since it can inhibit the expression of mucin (Mucin-2) facilitating a stronger subsequent binding mediated by the direct interaction of different adhesins (flagellins, pilins and various outer membrane proteins (OMPs) such as IipA and OmpU, among other factors) with their receptors on epithelial cells (Goo et al., 2006; Lee et al., 2010). Bacteria could use all these adhesins and receptors to colonize a wound.

Concomitantly to the colonization, the pathogen has to resist the bactericidal action of multiple stressors (e.g. acid pH of the stomach; the microstatic agent lactoferrin [an iron chelator], multiple microcidal peptides, etc.) that are common to fish and human's immune defences. To resist these defences, *V. vulnificus* uses multiple mechanisms, some of which are controlled by iron through Fur and/or by temperature (Table 1). These include (i) the proteins CadA and CadB (a lysine/cadaverine anti-carrier and a lysine decarboxylase), which synthesize and excrete cadaverine to counteract external pH (Rhee et al., 2002); (ii) a number of enzymes which confer resistance to acid pH; (iii) a sialised LPS that confers resistance to microcidal peptides; (iv) narrower porins (such as OmpU) that prevent bile salts from entering the cell; (v) a high-affinity iron uptake system that depends on the siderophore vulnibactin and (v) several heme receptors (Okujo and Shinoda, 1996; Webster and Litwin, 2000; Pajuelo et al., 2014).

In conclusion, the colonization of tissues and wounds by contact with seawater or marine animals and even the intestinal colonization of fish would be clearly favoured at warm temperatures and under iron-restricted conditions, being iron restriction a natural condition in marine and mucosal environments (Gledhiir and Buck, 2012).

Local lesions and invasion

Once *V. vulnificus* colonizes the epithelium, it probably secretes multiple toxins and exoenzymes to grow from different carbon sources naturally present in the tissues and open wounds (Reverter et al., 2018; Chernyavskikh et al., 2019). Temperature controls multiple genes related to the metabolism and transport of amino acids, lipids, fatty acids, nucleosides and iron, including the biosynthesis of vulnibactin, which globally result in a higher rate of colonization at higher temperatures (Table 1) (Hernández Cabanyero et al., 2020). At the same time, these toxins and exoenzymes will cause local damage (e.g. cellulitis after wound infection) enhanced by a local inflammatory response. Among these products, RtxA1 toxin is one of the most relevant. This toxin is expressed very early in the infection process after contact of the bacteria with different types of eukaryotic cells (Kim et al., 2008; Lee et al., 2013). RtxA1 belongs to the family of Multifunctional.

Autoprocessing Repeat in Toxin (MARTX). MARTX toxins are high molecular weight modular proteins with preserved external modules (in particular, the N-terminal and C terminal regions) that form pores in the eukaryotic membrane, through which the central module of the toxin is translocated. The effector domains of the central module are released after activation of the cysteine protease domain (CPD) in the cytosol and attack eukaryotic cells by different specific mechanisms (Fig. 3). A host–pathogen interaction study focused in the gills (the main portal of entry for the pathogen) of fish infected by immersion with a zoonotic strain of *V. vulnificus* or its toxin-deficient mutant linked the toxin with an early upregulation of several inflammatory genes, local secretion of IL-8 and migration of dendritic cells expressing immune receptors (TLR2 and 5) to the infection site (Callol et al., 2015). Similarly, several studies with wild-type and mutant L1 strains in mice (animal model to study human vibriosis) showed that RtxA1 toxin together with cytolysin VvhA, a pore-forming toxin present in all strains of the species (Wright et al., 1985 ; Yamamoto et al., 1990), and probably Vvp protease, cause damage and inflammation of intestinal tissue and trigger a local inflammatory response that ends with the rupture of the tight junctions (Jeong and Satchell, 2012). RtxA1 can also act on endothelial cells, thus promoting the spread and invasion of internal organs (Murciano et al., 2015). Therefore, regardless of the host, the local damage caused by RtxA1 and other secondary toxins and exoenzymes would not only favour the growth of the pathogen and the production of lesions but also its entry into the bloodstream. In contrast to vvp, rtxA1 and vvhA are controlled by iron but not by temperature (Table 1). The regulation has been studied in some detail and involves three regulators, HlyU, Fur and SmcR, and, in the case of RtxA1, also the contact of the bacterium with the eukaryotic cell (Shao et al., 2011; Lee et al., 2013; Hernández-Cabanyero et al., 2019). A model of this regulatory network for both toxins is presented in Fig. 4 and will be commented in the last section.

Resistance to innate immunity in blood

Once in the bloodstream, only the strains capable of expressing the adequate mechanism to resist the host innate immunity will survive and cause septicemia. In this process, it is very important the bacterial response to iron concentration in blood and, in case of fish, to environmental temperature (as they are poikilothermic animals). Innate blood defences that are common to all vertebrates involve humoral and cellular factors such as microcidal peptides, transferrin (an iron chelator equivalent to lactoferrin in the mucosa) and serum complement (a series of proteins that once activated destroy the pathogen or mark it for later phagocytosis by different types of cells that can also secrete microcidal products such as reactive oxygen species and nitric oxide) (Weinberg, 2009; Kaufmann and Dorhoi, 2016; Uribe-Querol and Rosales, 2017).

Figure 4 summarizes the main regulators and external signals involved in septicemia and sepsis in humans and fish according to Hernández-Cabanyero et al. (2019). When the bacterium invades the bloodstream of a healthy fish it detects a reduction in iron concentration and triggers an anaerobic metabolism of amino compounds based on nitrite/nitrate respiration that concomitantly activates protection against nitric oxide (Table 1 and Fig. 4) (Hernández-Cabanyero et al., 2019). The bacterium also produces a protective envelope rich in the high/medium molecular weight form of the LPS and two iron-regulated outer membrane proteins encoded in the virulence plasmid pVvBt2: the fish transferrin binding protein (Ftbp), a receptor for transferrin from eel and other teleostei (Pajuelo et al., 2015), and the fish phagocytosis and complement resistance protein (Fpcrp [formerly Vep07]), a multifunctional protein that confers specific resistance to eel complement and phagocytosis (Hernández-Cabanyero et al., 2019) (Fig. 4). In addition, if the fish is in a warm environment, *V. vulnificus* upregulates multiple genes encoding antioxidant enzymes, enzymes involved in LPS biosynthesis and membrane regeneration and, most importantly, the plasmid gene *ftbp* together with

genes involved in additional iron uptake mechanisms (Table 1) (Hernández-Cabanyero et al., 2020). Among the different genes mentioned, the most relevant are those encoded in pVvBt2 since their elimination implies the loss of virulence for fish (Lee et al., 2008; Pajuelo et al., 2015; Hernández-Cabanyero et al., 2019). In consequence, iron restrictions as well as warm temperature are factors that determine the survival of the bacterium in the fish blood and the septicemia. Interestingly, homologous genes of *ftbp* and *fpcrp* with almost 100% identity and coverage are present in a conjugated virulence plasmid of *V. harveyi* (pVh1) (<https://www.ncbi.nlm.nih.gov/nuccore/HM752267>), and unexpectedly, in other *V. vulnificus* strains belonging to other lineages, not previously associated with fish diseases but associated with human infection after ingestion of raw shellfish (formerly, L1 strains) (unpublished results). This result supports the conclusions reached by the above-mentioned genomic works on the role of fish farms as hot spots for genetic exchanges and, consequently, the emergence of new groups potentially pathogenic to fish. Furthermore, phylogenetic analysis of both proteins divides the strains into groups supposedly related to host specificity, indicating that a change in a few amino acids could adapt the protein to the immune system of a new host (unpublished results).

In case of humans, the iron concentration in blood is also absolutely critical although in an opposite sense. *V. vulnificus* can use a sialic-acid-like LPS plus the pump TrkA to resist in serum but the only virulence factor that is absolutely essential to survive and grow in human serum is the capsule (Carda-Diguez et al., 2018). The capsule of *V. vulnificus* confers resistance to both human complement and phagocytosis (Amaro et al., 1994; Williams et al., 2014; Carda-Diguez et al., 2018) and its biosynthesis is controlled by iron (Pajuelo et al., 2016). In fact, capsule biosynthesis is upregulated under iron excess conditions in human serum (Hernández-Cabanyero et al., 2019), which would explain why the main risk factor predisposing humans to septicemia is iron. Concomitantly, iron excess in human serum triggers in bacteria an anaerobic metabolism based on glycogen(maltose)-related compounds utilization and organic-compounds respiration (such as fumarate), which would provide to the bacterium with monomers for capsule biosynthesis (Table 1; Fig. 4) (Hernández-Cabanyero et al., 2019). Finally, the pathogen can also use the toxin RtxA1 to kill phagocytes and resist the phagocytosis (Lo et al., 2011; Chen et al., 2017). Interestingly, this toxin is overexpressed under iron excess in human serum (Fig. 4), which would increase the resistance of the pathogen to the innate immunity favouring septicemia.

Toxins and sepsis

Sepsis caused by *V. vulnificus* is mainly due to the action of the immune system against the bacterium that, eventually, causes severe injury to tissues and organs, including the endothelium. Neutrophils, macrophages and probably endothelial cells are the master cells in articulating the early response against pathogens in the blood and internal organs from both humans and fish. In accordance, the proinflammatory cytokines (CKs, interleukins IL-1 β , IL-6, IL-8), and tumour necrosis factor alpha (TNF- α) have been detected in the blood of diseased hosts with sepsis caused by *V. vulnificus* both humans and fish (Shin et al., 2002; Hernández-Cabanyero et al., in preparation). There is an apparent exception since patients with chronic liver diseases that should be predisposed to sepsis by *V. vulnificus* are paradoxically deficient in these CKs (Powell et al., 2003). Probably, the key is not the quantity but the quality of the immune response. Thus, the hypothesis is that *V. vulnificus* induces a dysregulated and unbalanced immune response in which these and other CKs and chemokines are involved.

Recently, RtxA1 produced by *V. vulnificus* zoonotic strains (all of them related to infections by contact) has been clearly associated with an early and unbalanced CK-storm by using an animal model of sepsis

(Murciano et al., 2017). The animals were infected either with the wild-type strain or with a deficient mutant in the toxin and the early differential immune response was analysed. The toxin was clearly related to an unbalanced immune response based on the upregulation of genes for multiple CKs (*il3*, *il4*, *il6*, *il7*, *il17a*, *csf2*, and *lta*), IFN-related genes (*ifna2*, *ifnb1*, *ifnar1*, and *ifngr1*), inflammasome components (*nlr4* and *casp1*) and transcription factors (*fos*, *jun*, *junB*, *nfkb1*, and *rela*) (Murciano et al., 2017). A similar result was also found in eels with the toxin linked to an early upregulation of CKs (*il1b*, *il1r*, *il6*, *il6r*, *il8*, *il10*, *il17* and *il20*), chemokine and their receptors (*ccl19*, *ccl4*, *cxc10*, *cmlkr4* and *cxcr4*), IFN-related genes (*ifnc*) and signal transducers and transcriptional factors (*traf3*, *irf2*, *mapks*, *nfkb*, *klf13* and *junB*), altogether leading to an early atypical immune response that probably triggers sepsis and death in the animals (Hernández-Cabanyero et al., in preparation). In addition, LPS (a potent inducer of TNFa) (Mayer et al., 2014), IlpA (Goo et al., 2007) and the capsule (Powell et al., 1997) can additively act as strong inducers of CK and chemokine production, contributing to death by sepsis.

Interestingly, VvhA could also participate in sepsis by acting additively with RtxA1 (Jeong and Satchell, 2012; Hernández-Cabanyero et al., 2019). In fact, *vvhA* and *rtxA1* are expressed early in artificial blood from risk patients and fish as well as *in vivo* in eels and mice (Murciano et al., 2017; Hernández-Cabanyero et al., 2019). As mentioned above, the transcription of *vvhA* and *rtxA1* is regulated by a complex process, partially known involving Fur, SmcR and HlyU (Fig. 4). The final result is that, although both genes are differentially regulated in serum (Table 1), of the two, only *rtxA1* seems to respond directly to iron levels, being overexpressed in excess conditions in this metal (Hernández-Cabanyero et al., 2019). Transcriptomic and phenotypic evidence support that VvhA would be produced earlier in fish blood, then the toxin would lyse the erythrocytes creating an iron-enriched microenvironment that together with contact with the eukaryotic cell would activate the production and secretion of RtxA1. In the case of the blood of risk patients, VvhA would be produced together with RtxA1 from the first hour of infection, probably with both toxins acting additively *in vivo* (Hernández-Cabanyero et al., 2019). Figure 5 presents a model of sepsis that summarizes all these findings. In conclusion, the iron content of the blood is one of the main external signals that trigger the virulent phenotype in *V. vulnificus*, a phenotype that would be specifically adapted to each host and condition.

Concluding remarks

V. vulnificus is an aquatic bacterium whose life cycle is controlled by iron and temperature. This bacterium is a fish pathogen that accidentally infects humans through multiple routes causing sepsis and death at risk patients. In general terms, iron deficiency and warm temperatures trigger the colonization of the mucosal surfaces of fish, as well as the colonization of human wounds. In fish blood, the septicemic condition is favoured by the iron restriction that will trigger the expression of a plasmid encoded system of survival and, concomitantly, the toxins responsible for death from sepsis. In human blood, the septicemic condition is favoured by a high iron content, which will trigger the production of both a protective capsule and the toxins that cause death by sepsis. The species should also be considered as a zoonotic agent of concern, especially by fish farmers and consumers, and fish farms as engines that accelerate the evolution of this species and the emergence of new virulent variants. In fact, fish farms have already favoured the exchange of the virulence plasmid between strains of different lineages within the species and even between different species. Finally, this pathogen is considered a biological marker of climate change because the number of human and animal infections it causes is increasing proportionally to the increase in seawater temperature (Vezzulli et al., 2013; Baker-Austin et al., 2017). The reason for this increase is that *V. vulnificus* is specially adapted to respond to temperature by activating genes involved in colonization and resistance in blood, which makes it a pathogen of concern in the face of global warming.

Fig. 1. Clinical signs of human and fish vibriosis caused by *Vibrio vulnificus*. Human vibriosis: A patient showing typical clinical signs of primary and secondary sepsis caused by *V. vulnificus*: swelling, erythema, development of vesicles or bullae and tissue necrosis (picture credits to Dr. Ching-Chuan Liu, Department of Paediatrics, National Cheng Kung University Hospital, College 2653 of Medicine, National Cheng Kung University, Tainan City, Taiwan). Eel vibriosis: hemorrhagic septicemia caused by contact (A) or by the oral route (B) (picture credits to C. Amaro and B. Fouz).

Human vibriosis



Fish vibriosis

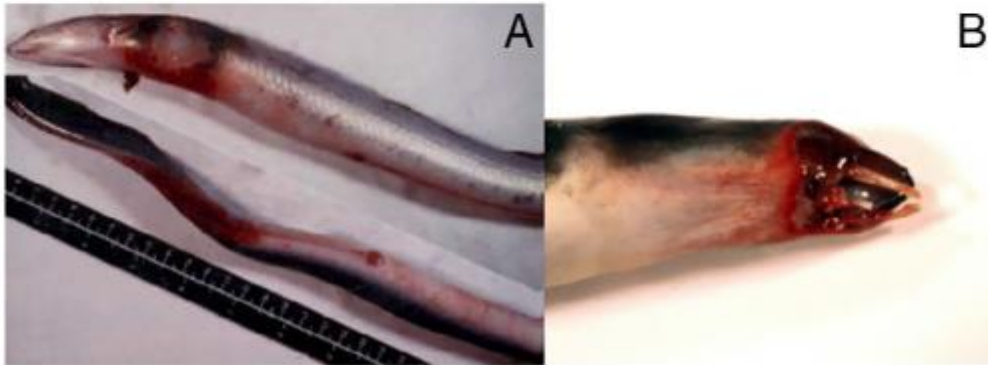


Fig. 2. The life cycle of *Vibrio vulnificus*: role of temperature and iron. This figure summarizes the life cycle of *V. vulnificus* and the role of temperature and iron concentration in the surrounding environment in determining its life strategy (Pajuelo et al., 2016; Hernández-Cabanyero et al., 2019, 2020). Temperature- and iron-controlled processes are surrounded by a blue or green line respectively, and the main genes involved are represented by different colours (see the head of the figure). Globally, a rise in temperature increases the transcription of genes related to metabolism, colonization (chemotaxis, flagellum biosynthesis, motility and the protease Vvp) and resistance to fish innate immunity (O-antigen biosynthetic genes and several plasmid genes) while iron controls genes involved in the same processes plus the two main toxins VvhA and RtxA1.

1. Survival in water. *V. vulnificus* survives in water either as a free-living cell or by forming biofilms on biotic or abiotic surfaces. Low temperatures induce the entrance of the bacterium in a 'dormant' state known as VBNC (viable but not culturable) state while warm temperatures activate resuscitation and biofilm dispersion.
2. Colonization of the host. Bacteria are attracted by blood and/or mucus from their susceptible hosts and colonize them, a process enhanced at a warm temperature and also controlled by iron. Bacteria can also be taken up by filtering organisms and these be ingested by humans.
3. Local lesions and invasion. Bacteria grow and produce toxins and exoenzymes that cause tissue damage and inflammation. From the colonized tissue, the pathogen invades the bloodstream of the host.
4. Resistance to innate immunity in blood and sepsis. To resist the innate immunity in human blood, the pathogen produces a capsule whose synthesis is increased under iron excess conditions in risk patients. To resist innate immunity in fish blood, the pathogen produces an outer membrane enriched in O-antigen plus two proteins (Fpcrp [fish phagocytosis and complement resistance protein] and Ftbp [fish transferrin binding protein]) encoded in the plasmid, whose synthesis is increased under iron starvation and at warm temperatures. Only the cells that resist the innate immunity multiply and secrete the toxins RtxA1 and VvhA that will cause the death of the host by a toxic sepsis, a complex process regulated by iron through several master regulators (demonstrated in mice by Murciano et al., 2017).
5. Transmission to new hosts and the environment. Diseased fish can infect humans (zoonosis) by contact and shed live bacteria in the water as well. The transmission of the pathogen is increased at warm temperatures.

Processes controlled by iron
 Processes controlled by temperature

Genes controlled by iron
 Genes controlled by temperature
 Genes controlled by iron and temperature

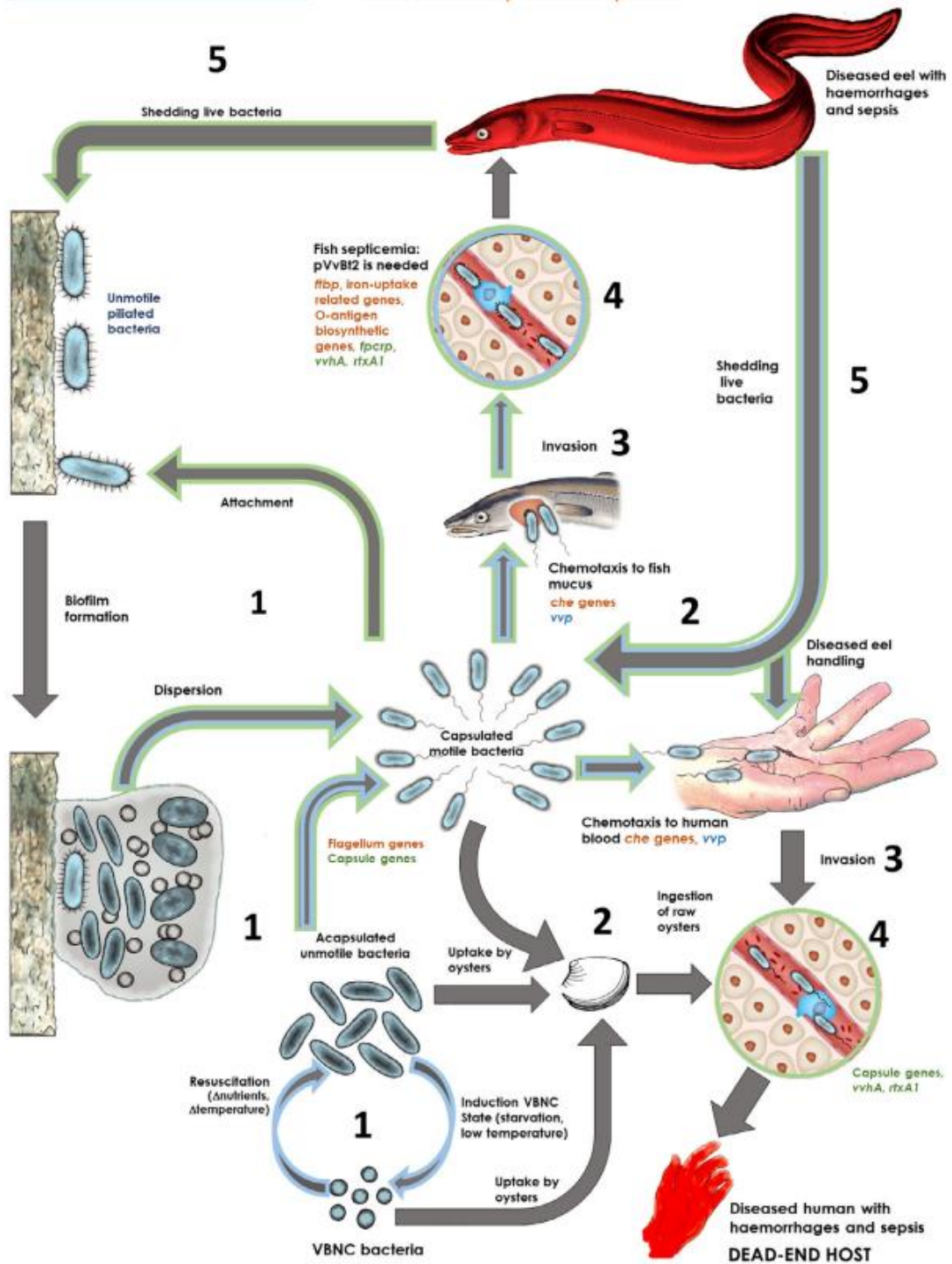
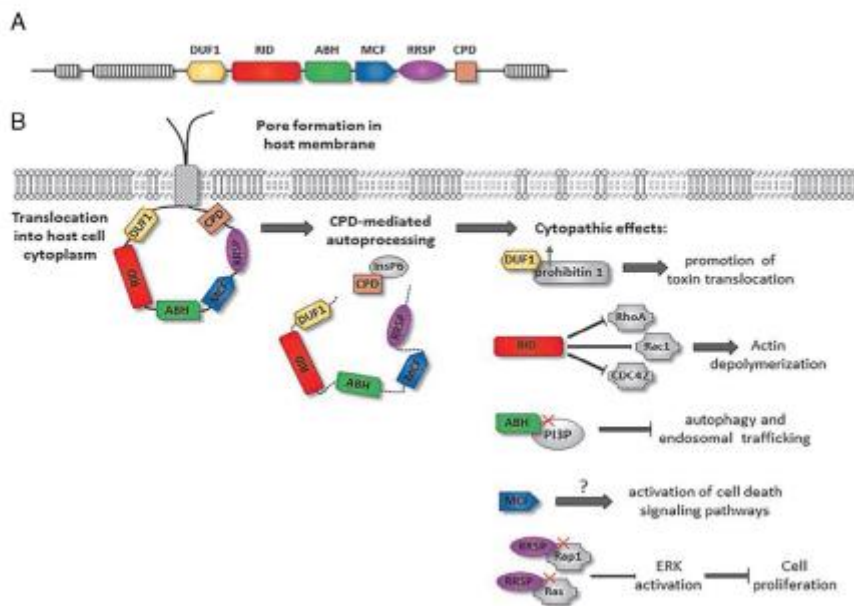


Fig. 3. Main *Vibrio vulnificus* RtxA1 toxins: Structure (A) and mechanism of action (B). RtxA11 is mainly produced by strains belonging to L1 and RtxA13 by the strains belonging to the zoonotic clonal-complex (L2). (A) The scheme shows the conserved external modules and the internal module, containing the effector domains represented in different colours. When the toxin is secreted, the external module is associated with the target cell membrane by forming a pore that allows the central module to be exposed to the cytosol. Then, the CPD is activated after binding the inositol hexakisphosphate (InsP6). Once activated, it catalyses the release of the rest of the internal domains that are responsible for the cytotoxicity towards eukaryotic cells. (B). The domain of unknown function (DUF1): It probably binds prohibitin-1 promoting the translocation of the toxin into the cell; Rho GTPase inhibitor domain (RID): It activates actin depolymerization, altering the cell cytoskeleton; Alpha/beta hydrolase domain (ABH): It binds inositol-3-phosphate inhibiting autophagy and endosomal trafficking; 'Makes caterpillars floppy' like domain (MCF): It induces depolarization of the mitochondrial membrane potential causing activation of cell death signalling pathways; Ras/Rap1 specific endopeptidase domain (RRSP): It suppresses the ERK-mitogen-activated protein kinase pathway, thus inhibiting cell proliferation; Actin cross-linking domain (ACD): It activates actin cross-linking. Images were taken from Ceccarelli et al. (2019) and Amaro et al. (2020).

RtxA1₁



RtxA1₃

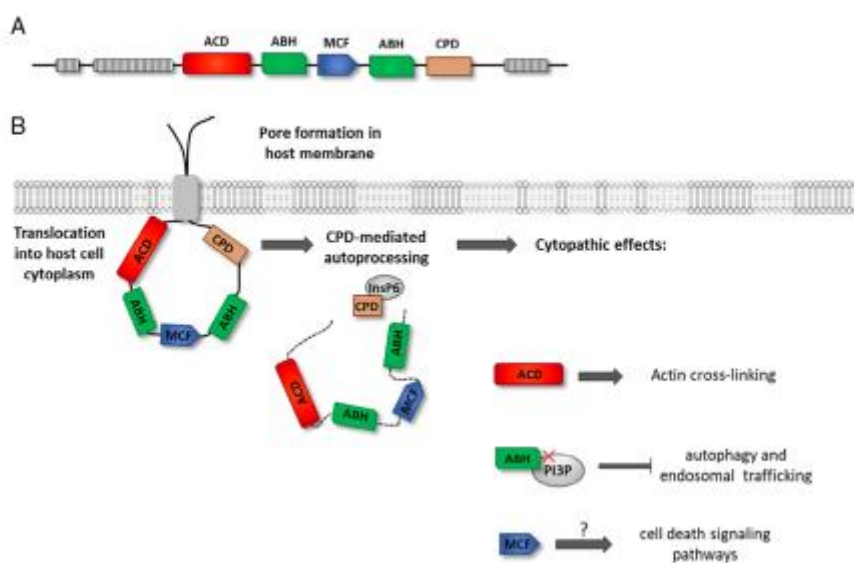


Fig. 4. Regulatory network, external stimulus and phenotype presented by zoonotic *Vibrio vulnificus* growing in serum: a holistic model per susceptible host. The models show the main regulators and sensors that would respond to the putative external signals in the serum as well as the resultant phenotype according to Hernández-Cabanyero et al. (2019). The regulators and sensors are presented as bubbles in red (upregulated in the transcriptome), green (downregulated in the transcriptome) and blue (predicted according to the literature but not found in the transcriptome) surrounded by line purple (belongs to both iron stimulon and Fur regulon (Pajuelo et al., 2016)), brown (belongs to the Fur regulon (Pajuelo et al., 2016)) or grey (belongs to the iron stimulon (Pajuelo et al., 2016)). Direct/indirect interaction between regulators and the predicted bacterial phenotype are shown as lines (confirmed results) or dotted lines. According to this model, *vvhA* (a gene mainly controlled by HlyU) would be upregulated under iron starvation by an unknown mechanism. Image modified from Hernández-Cabanyero et al. (2019)

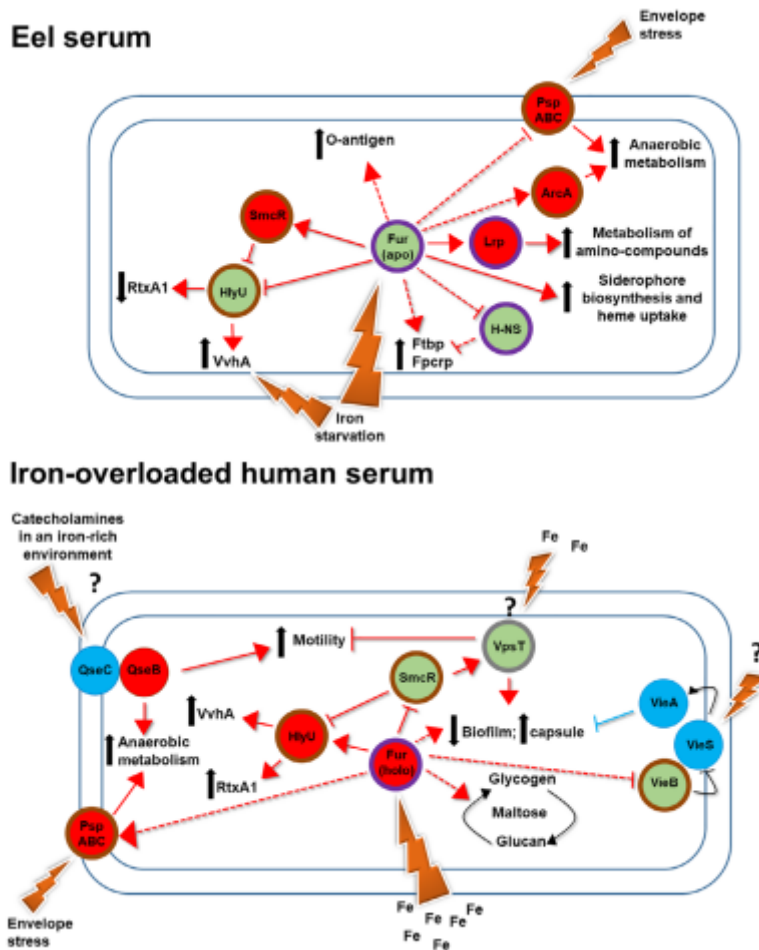


Fig. 5. Main virulence- and survival-related factors expressed by *Vibrio vulnificus* in host blood and their predicted function in septicemia and sepsis. Model in eel. In eel-blood, *V. vulnificus* cells detect iron-starvation and express an adapted external envelope that contains two plasmid OMPs, a receptor for eel transferrin (Ftbp) and a protective protein against eel complement and phagocytosis (Fpcrp), embedded in an LPS enriched in the high/medium molecular weight part of the O-antigen (1). As a consequence, the bacteria resist in serum and multiply, secreting VvhA (yellow balls), a cytotoxin that lyses red blood cells (nucleated red cells), which are very abundant in eel-blood (approximately 10^9 cells ml^{-1}), which in turn, liberate haemoglobin creating a microenvironment rich in iron as heme (2). In these conditions, and after contact with blood cells, the RtxA1 (in grey) is produced and secreted, entering into the eukaryotic cell (3) and causing a hypothetic cytokine storm, by unknown signalling cascades (4). Model in human. In the blood from a risk patient (iron-overloaded blood), the *V. vulnificus* cells detect iron-excess (red balls) and express an adapted external envelope enriched in a capsule that protects them against both complement and phagocytosis (1). The protected bacteria multiply and produce both VvhA (yellow ball) and RtxA1 (grey bars), which collaborate in cellular destruction (3). When RtxA1 interacts with the appropriate immune cell, it triggers a cytokine storm by inducing the overexpression of inflammation mediators as described by Murciano et al. (2017). Image modified from Hernández-Cabanyero et al. (2019).

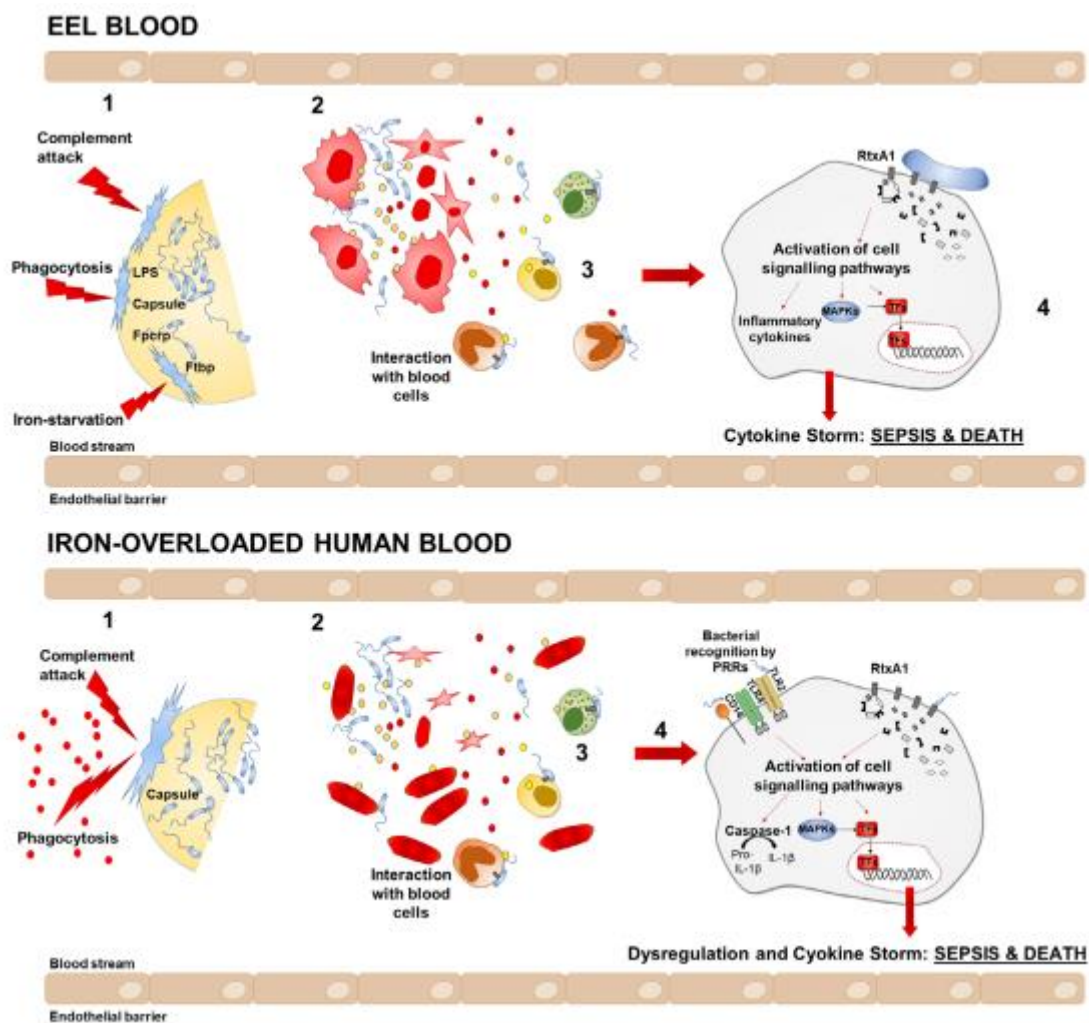


Table 1. Selection of genes controlled by iron and/or temperature that are involved in different phases of the life cycle of *V. vulnificus* according to Pajuelo et al. (2016) and Hernández-Cabanyero et al. (2019, 2020).

Gene(s) ^a	Iron ^b	Fur dependence ^c	Temperature ^d	Putative function/process ^e	Main role in the life cycle ^f
Aerobactin related genes	Downregulated	Yes	–	Aerobactin biosynthesis and transport	2
<i>aphC</i>	Upregulated	Yes	–	Oxidative stress resistance (VBNC state)	1
<i>che</i> genes	Upregulated	Yes	Upregulated	Chemotaxis related	1, 2
<i>cps</i> genes	Upregulated	Yes	–	Capsule biosynthesis	1, 4
Ferric iron ABC transporters	Downregulated	Yes	Upregulated	Ferric iron transport	2
Ferrous iron ABC transporters	Downregulated	Yes	Upregulated	Ferrous iron transport	2
<i>fla</i> genes	Upregulated	Yes	Upregulated	Flagellin proteins	1, 2
<i>flg</i> genes	Upregulated	Yes	Upregulated	Flagellar hook proteins	1, 2
<i>flh</i> genes	Upregulated	Yes	–	Flagellar biosynthesis proteins	1, 2
<i>flp (cpa)</i> genes	Downregulated	Yes	–	Flp pilus assembly	1, 2
<i>fpcrp</i>	Downregulated	Yes	–	Fish phagocytosis and complement resistance protein (Vep07)	4
<i>ftbp</i>	Downregulated	Yes	Upregulated	Fish transferrin binding protein (Vep20)	4
<i>galE</i>	–	–	Upregulated	O-antigen biosynthesis	4
Glycogen (maltose) related compounds	Upregulated in iron overloaded human serum	–	Upregulated	Glycogen metabolism	4
Glutathione synthesis and transport	–	–	Upregulated	Resistance to oxidative stress	1, 4
<i>lptA, lptB</i>	–	Yes	Upregulated	LPS biosynthesis	2
<i>mot</i> genes	Upregulated	Yes	Upregulated	Flagellar motor rotation protein	1
<i>msha</i> genes	Downregulated	Yes	Upregulated	MSHA pilus biosynthesis	1, 2
<i>nnrs</i>	Downregulated	Yes	–	Resistance to nitrosative stress	1, 4
<i>ompU</i>	Downregulated	Yes	–	Resistance to microcidal peptides	2
Phosphoglycerol transferases and dehydrogenases	–	–	Upregulated	Membrane regeneration (phospholipid biosynthesis)	4
<i>plsX, plsY</i>	–	–	Upregulated	Membrane regeneration (phospholipid biosynthesis)	4
<i>rtxA</i> ^g	Upregulated	–	–	MARTX toxin	3, 4, 5
<i>sodA</i>	Downregulated	Yes	–	Oxidative stress resistance	2
Superoxide dismutases	–	–	Upregulated	Resistance to oxidative stress	3
<i>tad</i> genes	Downregulated	Yes	Upregulated	Flp pilus assembly	1
<i>trkA</i>	–	Yes	Yes	Potassium uptake	4
Vulnibactin-related genes	Downregulated	Yes	Upregulated	Vulnibactin biosynthesis and transport	2
<i>vvhA</i> ^g	Downregulated	No	–	Cytolysin	3, 5
<i>vvp</i>	–	–	Enhanced activity	Protease activity	2
<i>waaL</i>	Downregulated	Yes	–	LPS Lipid-A core O-antigen ligase	3
<i>wza, wcb, wzc</i>	Downregulated	Yes	–	LPS biosynthesis	2, 3
Regulators					
<i>arcA</i>	No	Yes	Downregulated	Repressor for aerobic metabolism	1, 2, 4
<i>fleN</i>	Upregulated	Yes	Upregulated	Flagellar synthesis regulator	1, 2
<i>kdgR</i>	Downregulated	Yes	–	Repressor for amino sugars metabolism	4
<i>norR</i>	No	No	Upregulated	Anaerobic nitric oxide reductase transcription regulator	1, 2, 4
<i>nsrR</i>	Upregulated	Yes	Upregulated	Repressor of resistance to nitrosative stress	1, 2, 4

^a Identified differentially expressed genes in iron/or temperature stimulons are indicated.

^b Gene regulation in iron excess conditions (Pajuelo et al., 2016).

^c Gene expression depends on Fur (Pajuelo et al., 2016).

^d Gene regulation above 25°C (Hernández-Cabanyero et al., 2020).

^e Putative function for the gene and related process.

^f The role of each gene in the pathogen's life cycle is indicated. The stages of the life cycle are indicated by a number code: 1, survival in water; 2, colonization of the host; 3, local lesions and invasion; 4, resistance to innate immunity in the blood; 5, toxins and sepsis.

^g The regulation of the toxins RtxA1 and VvhA have been studied in some detail. It depends on iron content through the interaction of three regulators HlyU, Fur and SmcR and, in the case of RtxA1, also on the contact of the bacterium with the eukaryotic cell (Hernández-Cabanyero et al., 2019; Shao et al., 2011; Kim et al., 2013) (see Fig. 5).

Acknowledgements

This work has been financed by grants AGL2017-87723-P (co-funded with FEDER funds) from the Ministry of Science, Innovation and Universities (Spain) and AICO/2018/123 from Generalitat Valenciana (Spain). Hernández-Cabanyero C has been financed by grant BES-2015-073117, an FPI grant from the Ministry of Science, Innovation and Universities (Spain).

References

- Amaro, C., and Biosca, E.G. (1996) *Vibrio vulnificus* biotype 2, pathogenic for eels, is also an opportunistic pathogen for humans. *Appl Environ Microbiol* 62: 1454–1457.
- Amaro, C., Biosca, E.G., Fouz, B., Alcaide, E., and Esteve, C. (1995) Evidence that water transmits *Vibrio vulnificus* biotype 2 infections to eels. *Appl Environ Microbiol* 61: 1133–1137.
- Amaro, C., Biosca, E.G., Fouz, B., Toranzo, A.E., and Garay, E. (1994) Role of iron, capsule and toxins in the pathogenicity of *Vibrio vulnificus* biotype 2 for mice. *Infect Immun* 62: 759–763.
- Amaro, C., Fouz, B., Sanjuán, E., and Romalde, J. (2020) Vibriosis. In *Climate Change and Infectious Fish Diseases*. Whoo, P.T.K., Leong, J.A., and Buchmann, K. (eds). Oxfordshire, England: CABI.
- Amaro, C., Sanjuán, E., Fouz, B., Pajuelo, D., Lee, C., Hor, L., and Barrera, R. (2015) The fish pathogen *Vibrio vulnificus* biotype 2: epidemiology, phylogeny and virulence factors involved in warm-water vibriosis. *Microbiol Spectr* 3.
- Baker-Austin, C., and Oliver, J.D. (2018) *Vibrio vulnificus*: new insights into a deadly opportunistic pathogen. *Environ Microbiol* 20: 423–430.
- Baker-Austin, C., Stockley, L., Rangdale, R., and Martinez Urtaza, J. (2010) Environmental occurrence and clinical impact of *Vibrio vulnificus* and *Vibrio parahaemolyticus*: a European perspective. *Environ Microbiol Rep* 2: 7–18.
- Baker-Austin, C., Trianes, J., Gonzalez-Escalona, N., and Martinez-Urtaza, J. (2017) Non-cholera Vibrios: the microbial barometer of climate change. *Trends Microbiol* 25: 76–84.
- Biosca, E.G., Amaro, C., Esteve, C., Alcaide, E., and Garay, E. (1991) First record of *Vibrio vulnificus* biotype 2 from diseased European eel, *Anguilla Anguilla*. *J Fish Dis* 14: 103–109.
- Biosca, E.G., Collado, R.M., Oliver, J.D., and Amaro, C. (1999) Comparative study of biological properties and electrophoretic characteristics of lipopolysaccharide from Eel-virulent and Eel-avirulent *Vibrio vulnificus* strains. *Appl Environ Microbiol* 65: 856–858.
- Biosca, E.G., Oliver, J.D., and Amaro, C. (1996) Phenotypic characterization of *Vibrio vulnificus* biotype 2, a lipopolysaccharide-based homogeneous O serogroup within *Vibrio vulnificus*. *Appl Environ Microbiol* 62: 918–927.
- Bisharat, N., Agmon, V., Finkelstein, R., Raz, R., BenDror, G., Lerner, L., et al. (1999) Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel. *Lancet* 354: 1421–1424.
- Bisharat, N., Amaro, C., Fouz, B., Llorens, A., and Cohen, D.I. (2007) Serological and molecular characteristics of *Vibrio vulnificus* biotype 3: evidence for high clonality. *Microbiology* 153: 847–856.

- Bisharat, N., Cohen, D.I., Harding, R.M., Falush, D., Crook, D.W., Peto, T., and Maiden, M.C. (2005) Hybrid *Vibrio vulnificus*. *Emerg Infect Dis* 11: 30–35.
- Broza, Y.Y., Danin-Poleg, Y., Lerner, L., Valinsky, L., Broza, M., and Kashi, Y. (2009) Epidemiologic study of *Vibrio vulnificus* infections by using variable number tandem repeats. *Emerg Infect Dis* 15: 1282–1285.
- Broza, Y.Y., Raz, N., Lerner, L., Danin-Poleg, Y., and Kashi, Y. (2012) Genetic diversity of the human pathogen *Vibrio vulnificus*: a new phylogroup. *Int J Food Microbiol* 153: 436–443.
- Callol, A., Pajuelo, D., Ebbesson, L., Teles, M., MacKenzie, S., and Amaro, C. (2015) Early steps in the European eel (*Anguilla Anguilla*)-*Vibrio vulnificus* interaction in the gills: role of the RtxA13 toxin. *Fish Shellfish Immunol* 43: 502–509.
- Carda-Diéguez, M., Ghai, R., Rodríguez-Valera, F., and Amaro, C. (2017) Wild eel microbiome reveals that skin mucus of fish could be a natural niche for aquatic mucosal pathogen evolution. *Microbiome* 5: 162.
- Carda-Diguez, M., Silva-Hernandez, F., Hubbard, T., Chao, M., Waldor, M., and Amaro, C. (2018) Comprehensive identification of *Vibrio vulnificus* genes required for growth in human serum. *Virulence* 9: 981–993.
- Casper-lindley, C., and Yildiz, F.H. (2004) VpsT is a transcriptional regulator required for expression of vps biosynthesis genes and the development of rugose colonial morphology in *Vibrio cholerae* O1 El Tor. *J Bacteriol* 186: 1574–1578.
- Ceccarelli, D., Amaro, C., Romalde, J., Suffredini, E., and Vezzulli, L. (2019) *Vibrio* species. In *Food Microbiology: Fundamentals and Frontiers*, Doyle, M., Diez-González, F., and Hill, C. (eds). Washington, DC: ASM Press.
- Chen, C.-L., Chien, S.-C., Leu, T.-H., Harn, H.I.-C., Tang, M.-J., and Hor, L.-I. (2017) *Vibrio vulnificus* MARTX cytotoxin causes inactivation of phagocytosis-related signaling molecules in macrophages. *J Biomed Sci* 24: 58.
- Chen, C.Y., Chao, C.B., and Bowser, P.R. (2006) Infection of tilapia *Oreochromis* sp. by *Vibrio vulnificus* in freshwater and low-salinity environments. *J World Aquacult Soc* 37: 82–88.
- Chernyavskikh, S.D., Borodaeva, Z., Borisovskiy, I.P., Ostapenko, S.I., and Galtseva, O.A. (2019) Blood protein spectrum in representatives of the fish superclass. *EurAsian J Biosci* 13: 979–981.
- Cohen, A.L.V., Oliver, J.D., DePaola, A., Feil, E.J., and Boyd, E.F. (2007) Emergence of a virulent clade of *Vibrio vulnificus* and correlation with the presence of a 33-kilobase genomic Island. *Appl Environ Microbiol* 73: 5553–5565.
- Dalsgaard, A., Frimodt-Moller, N., Bruun, B., Hoi, L., and Larsen, J.L. (1996) Clinical manifestations and molecular epidemiology of *Vibrio vulnificus* infections in Denmark. *Eur J Clin Microbiol Infect Dis* 15: 227–232.
- Danin-Poleg, Y., Elgavish, S., Raz, N., Efimov, V., and Kashi, Y. (2013) Genome sequence of the pathogenic bacterium *Vibrio vulnificus* biotype 3. *Genome Announc* 1: e0013613.
- Efimov, V., Danin-Poleg, Y., Raz, N., Elgavish, S., Linetsky, A., and Kashi, Y. (2013) Insight into the evolution of *Vibrio vulnificus* biotype 3's genome. *Front Microbiol* 4: 1–6.

- Elgaml, A., and Miyoshi, S.I. (2017) Regulation systems of protease and hemolysin production in *Vibrio vulnificus*. *Microbiol Immunol* 61: 1–11.
- Esteve, C., Alcaide, E., Herraiz, S., Canals, R., Merino, S., and Tomás, J.M. (2007) First description of nonmotile *Vibrio vulnificus* strains virulent for eels. *FEMS Microbiol Lett* 266: 90–97.
- Farmer, J.J., 3rd. (1979) *Vibrio* (“Benecke”) *vulnificus*, the bacterium associated with sepsis, septicaemia and the sea. *Lancet* 314: 903. Fouz, B., and Amaro, C. (2002) Isolation of a new serovar of *Vibrio vulnificus* pathogenic for eels cultured in freshwater farms. *Aquaculture* 217: 677–682.
- Fouz, B., Larsen, J.L., and Amaro, C. (2006) *Vibrio vulnificus* serovar A: an emerging pathogen in European anguilliculture. *J Fish Dis* 29: 285–291.
- Fouz, B., Llorens, A., Valiente, E., and Amaro, C. (2010) A comparative epizootiologic study of the two fish-pathogenic serovars of *Vibrio vulnificus* biotype 2. *J Fish Dis* 33: 383–390.
- Gledhiir, M., and Buck, K.N. (2012) The organic complexation of iron in the marine environment: a review. *Front Microbiol* 3: 1–17.
- González-Escalona, N., Jaykus, L.-A., and DePaola, A. (2007) Typing of *Vibrio vulnificus* strains by variability in their 16S-23S rRNA intergenic spacer regions. *Foodborne Pathog Dis* 4: 327–337.
- Goo, S.Y., Lee, H.J., Kim, W.H., Han, K.L., Park, D.K., Lee, H.J., et al. (2006) Identification of OmpU of *Vibrio vulnificus* as a fibronectin-binding protein and its role in bacterial pathogenesis. *Infect Immun* 74: 5586–5594.
- Goo, S.Y., Soo, H.Y., Kim, W.H., Lee, K.H., and Park, S.J. (2007) *Vibrio vulnificus* IlpA-induced cytokine production is mediated by toll-like receptor 2. *J Biol Chem* 282: 27647–27658.
- Hernández-Cabanyero, C., Lee, C.-T., Tolosa-Enguis, V., Sanjuán, E., Pajuelo, D., Reyes-López, F., et al. (2019) Adaptation to host in *Vibrio vulnificus*, a zoonotic pathogen that causes septicemia in fish and humans. *Environ Microbiol* 21: 3118–3139.
- Hernández-Cabanyero, C., Sanjuán, E., Fouz, B., Pajuelo, D., Vallejos-Vidal, E., Reyes-López, F.E., and Amaro, C. (2020) The effect of the environmental temperature on the adaptation to host in the zoonotic pathogen *Vibrio vulnificus*. *Front Microbiol* 11: 1–17.
- Høi, L., Larsen, J.L., Dalsgaard, I., and Dalsgaard, A. (1998) Occurrence of *Vibrio vulnificus* biotypes in Danish marine environments. *Appl Environ Microbiol* 64: 7–13.
- Horseman, M.A., and Surani, S. (2011) A comprehensive review of *Vibrio vulnificus*: an important cause of severe sepsis and skin and soft-tissue infection. *Int J Infect Dis* 15: e157–e166.
- Jang, K.K., Gil, S.Y., Lim, J.G., and Choi, S.H. (2016) Regulatory characteristics of *Vibrio vulnificus* gbpA gene encoding a mucin-binding protein essential for pathogenesis. *J Biol Chem* 291: 5774–5787.
- Jeong, H.G., and Satchell, K.J.F. (2012) Additive function of *Vibrio vulnificus* MARTXVv and VvhA cytolytins promotes rapid growth and epithelial tissue necrosis during intestinal infection. *PLoS Pathog* 8: e1002581.
- Jones, M.K., and Oliver, J.D. (2009) *Vibrio vulnificus*: disease and pathogenesis. *Infect Immun* 77: 1723–1733. Joseph, L.A., and Wright, A.C. (2004) Expression of *Vibrio vulnificus* capsular polysaccharide inhibits biofilm formation. *J Bacteriol* 186: 889–893.

- Kaspar, C.W., and Tamplin, M.L. (1993) Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. *Appl Environ Microbiol* 59: 2425–2429.
- Kaufmann, S.H.E., and Dorhoj, A. (2016) Molecular determinants in phagocyte-bacteria interactions. *Immunity* 44: 476–491.
- Kim, H.I., Wen, Y., Son, J.-S., Lee, K.-H., and Kim, K.S. (2013) The fur-iron complex modulates expression of the quorum-sensing master regulator, smcR, to control expression of virulence factors in *Vibrio vulnificus*. *Infect Immun*, 81: 2888–2898.
- Kim, Y.R., Lee, S.E., Kook, H., Yeom, J.A., Na, H.S., Kim, S. Y., et al. (2008) *Vibrio vulnificus* RTX toxin kills host cells only after contact of the bacteria with host cells. *Cell Microbiol* 10: 848–862.
- Koton, Y., Gordon, M., Chalifa-Caspi, V., and Bisharat, N. (2015) Comparative genomic analysis of clinical and environmental *Vibrio vulnificus* isolates revealed biotype 3 evolutionary relationships. *Front Microbiol* 5: 1–10.
- Lee, H.J., Kim, J.A., Lee, M.A., Park, S.J., and Lee, K.H. (2013) Regulation of haemolysin (VvhA) production by ferric uptake regulator (Fur) in *Vibrio vulnificus*: repression of vvhA transcription by Fur and proteolysis of VvhA by Fur-repressive exoproteases. *Mol Microbiol* 88: 813–826.
- Lee, K.J., Lee, N.Y., Han, Y.S., Kim, J., Lee, K.H., and Park, S.J. (2010) Functional characterization of the IipA protein of *Vibrio vulnificus* as an adhesin and its role in bacterial pathogenesis. *Infect Immun* 78: 2408–2417.
- Li, Z., Chen, H., Chen, X., Zhou, T., Zhao, L., Zhang, C., and Jin, W. (2012) Genome sequence of the human pathogenic bacterium *Vibrio vulnificus* type strain ATCC 27562. *J Bacteriol* 194: 6954–6955.
- Lo, H.R., Lin, J.H., Chen, Y.H., Chen, C.L., Shao, C.P., Lai, Y.C., and Hor, L.I. (2011) RTX toxin enhances the survival of *Vibrio vulnificus* during infection by protecting the organism from phagocytosis. *J Infect Dis* 203: 1866–1874.
- López-Pérez, M., Jayakumar, J.M., Haro-Moreno, J.M., Zaragoza-Solas, A., Reddi, G., Rodriguez-Valera, F., et al. (2019) Evolutionary model of cluster divergence of the emergent marine pathogen *Vibrio vulnificus*: from genotype to ecotype. *MBio* 10: 1–18.
- Marco-Noales, E., Biosca, E.G., and Amaro, C. (1999) Effects of salinity and temperature on long-term survival of the eel pathogen *Vibrio vulnificus* Biotype 2 (Serovar E). 65: 1117–1126.
- Marco-Noales, E., Milán, M., Fouz, B., Sanjuán, E., and Amaro, C. (2001) Transmission to eels, portals of entry, and putative reservoirs of *Vibrio vulnificus* Serovar E (Biotype 2). *Appl Environ Microbiol* 67: 4717–4725.
- Mayer, A.M.S., Hall, M.L., Holland, M., De Castro, C., Molinaro, A., Aldulescu, M., et al. (2014) *Vibrio vulnificus* MO6-24/O lipopolysaccharide stimulates superoxide anion, thromboxane B2, matrix metalloproteinase-9, cytokine and chemokine release by rat brain microglia in Vitro. *Mar Drugs* 12: 1732–1756.
- Menon, M.P., Yu, P.A., Iwamoto, M., and Painter, J. (2014) Pre-existing medical conditions associated with *Vibrio vulnificus* septicaemia. *Epidemiol Infect* 142: 878–881.
- Miyoshi, S. (2006) *Vibrio vulnificus* infection and metalloprotease. *J Dermatol* 33: 589–595.
- Miyoshi, S., Wakae, H., Tomochika, K., and Shinoda, S. (1997) Functional domains of a zinc metalloprotease from *Vibrio vulnificus*. *J Bacteriol* 179: 7606–7609.

- Miyoshi, S.I., Kawata, K., Tomochika, K.I., and Shinoda, S. (1999) The hemagglutinating action of *Vibrio vulnificus* metalloprotease. *Microbiol Immunol* 43: 79–82.
- Morrison, S.S., Williams, T., Cain, A., Froelich, B., Taylor, C., Baker-Austin, C., et al. (2012) Pyrosequencing-based comparative genome analysis of *Vibrio vulnificus* environmental isolates. *PLoS One* 7: e37553.
- Murciano, C., Hor, L.I., and Amaro, C. (2015) Host-pathogen interactions in *Vibrio vulnificus*: responses of monocytes and vascular endothelial cells to live bacteria. *Future Microbiol* 10: 471–487.
- Murciano, C., Lee, C.-T., Fernández-Bravo, A., Hsieh, T.-H., Fouz, B., Hor, L.I., and Amaro, C. (2017) MARTX toxin in the zoonotic serovar of *Vibrio vulnificus* triggers an early cytokine storm in mice. *Front Cell Infect Microbiol* 7: 1–19.
- Muroga, K., Jo, Y., and Nishibuchi, M. (1976) Pathogenic *Vibrio* isolated from cultured eels. Characteristics and taxonomic status. *Fish Pathog* 11: 141–145.
- Nilsson, W.B., Paranjpye, R.N., DePaola, A., and Strom, M. S. (2003) Sequence polymorphism of the 16S rRNA gene of *Vibrio vulnificus* is a possible indicator of strain virulence. *J Clin Microbiol* 41: 442–446.
- Okujo, N., and Shinoda, S. (1996) Involvement of vulnibactin in utilization of transferrin- iron by *Vibrio vulnificus* and exocellular protease and Lactoferrin-bound causing primary septicemia and severe wound infec- or LF has been lacking . In addition, there are conflicting putat. *Microbiol Immunol* 40: 595–598
- Oliver, J.D. (2015) The biology of *Vibrio vulnificus*. *Microbiol Spectr* 3: 1–10.
- Pajuelo, D., Hernández-Cabanyero, C., Sanjuan, E., Lee, C.-T., Silva-Hernández, F.X., Hor, L.I., et al. (2016) Iron and Fur in the life cycle of the zoonotic pathogen *Vibrio vulnificus*. *Environ Microbiol* 18: 4005–4022.
- Pajuelo, D., Lee, C.-T., Roig, F.J., Hor, L.I., and Amaro, C. (2015) Novel host-specific iron acquisition system in the zoonotic pathogen *Vibrio vulnificus*. *Environ Microbiol* 17: 2076–2089.
- Pajuelo, D., Lee, C.-T., Roig, F.J., Lemos, M.L., Hor, L.I., and Amaro, C. (2014) Host-nonspecific iron acquisition systems and virulence in the zoonotic serovar of *Vibrio vulnificus*. *Infect Immun* 82: 731–744.
- Paranjpye, R.N., and Strom, M.S. (2005) A *Vibrio vulnificus* type IV pilin contributes to biofilm formation, adherence to epithelial cells and virulence. *Infect Immun* 73: 1411–1422.
- Park, J.H., Cho, Y.J., Chun, J., Seok, Y.J., Lee, J.K., Kim, K. S., et al. (2011) Complete genome sequence of *Vibrio vulnificus* MO6-24/O. *J Bacteriol* 193: 2062–2063.
- Powell, J.L., Strauss, K.A., Wiley, C., Zhan, M., and Morris, J.G. (2003) Inflammatory cytokine response to *Vibrio vulnificus* elicited by peripheral blood mononuclear cells from chronic alcohol users is associated with biomarkers of cellular oxidative stress. *Infect Immun* 71: 4212–4216.
- Powell, J.L., Wright, A.C., Wasserman, S.S., Hone, D.M., and Morris, J.G. (1997) Release of tumor necrosis factor alpha in response to *Vibrio vulnificus* capsular polysaccharide in in vivo and in vitro models. *Infect Immun* 65: 3713–3718.
- Reverter, M., Tapissier-Bontemps, N., Lecchini, D., Banaigs, B., and Sasal, P. (2018) Biological and ecological roles of external fish mucus: a review. *Fishes* 3: 41. Rhee, J.E., Rhee, J.H., Ryu, P.Y., and Choi,

- S.H. (2002) Identification of the *cadBA* operon from *Vibrio vulnificus* and its influence on survival to acid stress. *FEMS Microbiol Lett* 208: 245–251.
- Roig, F.J., and Amaro, C. (2009) Plasmid diversity in *Vibrio vulnificus* biotypes. *Microbiology* 155: 489–497.
- Roig, F.J., González-Candelas, F., Sanjuán, E., Fouz, B., Feil, E.J., Llorens, C., et al. (2018) Phylogeny of *Vibrio vulnificus* from the analysis of the core-genome: implications for intra-species taxonomy. *Front Microbiol* 8: 1–13.
- Sadler, K. (1979) Effects of temperature on the growth and survival of the European eel, *Anguilla Anguilla* L. *J Fish Biol* 15: 499–507.
- Sanjuán, E., Fouz, B., Oliver, J.D., and Amaro, C. (2009) Evaluation of genotypic and phenotypic methods to distinguish clinical from environmental *Vibrio vulnificus* strains. *Appl Environ Microbiol* 75: 1604–1613.
- Sanjuán, E., González-Candelas, F., and Amaro, C. (2011) Polyphyletic origin of *Vibrio vulnificus* biotype 2 as revealed by sequence-based analysis. *Appl Environ Microbiol* 77: 688–695.
- Shao, C.P., Lo, H.R., Lin, J.H., and Hor, L.I. (2011) Regulation of cytotoxicity by quorum-sensing signaling in *Vibrio vulnificus* is mediated by SmcR, a repressor of hlyU. *J Bacteriol* 193: 2557–2565.
- Shin, S.H., Shin, D.H., Ryu, P.Y., Chung, S.S., and Rhee, J.H. (2002) Proinflammatory cytokine profile in *Vibrio vulnificus* septicemic patients' sera. *FEMS Immunol Med Microbiol* 33: 133–138.
- Tao, Z., Larsen, A.M., Bullard, S.A., Wright, A.C., and Arias, C.R. (2012) Prevalence and population structure of *Vibrio vulnificus* on fishes from the northern Gulf of Mexico. *Appl Environ Microbiol* 78: 7611–7618.
- Lee, C.-T., Amaro, C., Wu, K.M., Valiente, E., Chang, Y.F., Tsai, S.F., et al. (2008) A common virulence plasmid in biotype 2 *Vibrio vulnificus* and its dissemination aided by a conjugal plasmid. *J Bacteriol* 190: 1638–1648.
- Lee, C.-T., Pajuelo, D., Llorens, A., Chen, Y.H., Leiro, J.M., Padrós, F., et al. (2013) MARTX of *Vibrio vulnificus* biotype 2 is a virulence and survival factor. *Environ Microbiol* 15: 419–432.
- Tison, D.L., Nishibuchi, M., Greenwood, J.D., and Seidler, R.J. (1982) *Vibrio vulnificus* biogroup 2: new biogroup pathogenic for eels. *Appl Environ Microbiol* 44: 640–646.
- Uribe-Querol, E., and Rosales, C. (2017) Control of phago-cytosis by microbial pathogens. *Front Immunol* 8: 1–23.
- Valiente, E., Lee, C., Hor, L., Fouz, B., and Amaro, C. (2008) Role of the metalloprotease Vvp and the virulence plasmid pR99 of *Vibrio vulnificus* serovar E in surface colonization and fish virulence. *Environ Microbiol* 10: 328–338.
- Veenstra, J., Rietra, P.J., Stoutenbeek, C.P., Coster, J.M., de Gier, H.H., and Dirks-Go, S. (1992) Infection by an indole-negative variant of *Vibrio vulnificus* transmitted by eels. *J Infect Dis* 166: 209–210.
- Vezzulli, L., Colwel, R.R., and Pruzzo, C. (2013) Ocean warming and spread of pathogenic Vibrios in the aquatic environment. *Microb Ecol* 65: 817–825

- Vugia, D.J., Tabnak, F., Newton, A.E., Hernandez, M., and Griffin, P.M. (2013) Impact of 2003 state regulation on raw oyster-associated *Vibrio vulnificus* illnesses and deaths, California, USA. *Emerg Infect Dis* 19: 1276–1280.
- Wang, Z.-G., Wu, Z., Xu, S.-L., and Zha, J. (2012) Genome sequence of the human-pathogenetic bacterium *Vibrio vulnificus* B2. *J Bacteriol* 194: 7019.
- Webster, A.C.D., and Litwin, C.M. (2000) Cloning and characterization of *vuuA*, a gene encoding the *Vibrio vulnificus* ferric vulnibactin receptor. *Infect Immun* 68: 526–534.
- Weinberg, E.D. (2009) Iron availability and infection. *Biochim Biophys Acta - Gen Subj* 1790: 600–605.
- Whitesides, M.D., Oliver, J.D., and Carolina, N. (1997) Resuscitation of *Vibrio vulnificus* from the viable but Non culturable state. *Appl Environ Microbiol* 63: 1002–1005.
- Williams, T.C., Ayrapetyan, M., Ryan, H., and Oliver, J.D. (2014) Serum survival of *Vibrio vulnificus*: role of genotype, capsule, complement, clinical origin, and in situ incubation. *Pathogens* 3: 822–832.
- Wolf, P.W., and Oliver, J.D. (1992) Temperature effects on the viable but non-culturable state of *Vibrio vulnificus*. *FEMS Microbiol Lett* 101: 33–39.
- Wright, A.C., Morris, J.G., Maneval, D.R., Richardson, K., and Kaper, J.B. (1985) Cloning of the cytotoxin-hemolysin gene of *Vibrio vulnificus*. *Infect Immun* 50: 922–924.
- Yamamoto, K., Wright, A.C., Kaper, J.B., and Morris, J.G. (1990) The cytolysin gene of *Vibrio vulnificus*: sequence and relationship to the *Vibrio cholerae* El Tor hemolysin gene. *Infect Immun* 58: 2706–2709