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# Iron and Fur in the life cycle of the zoonotic pathogen *Vibrio vulnificus*

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## Summary

**In this study, we aimed to analyze the global response to iron in the broad-range host pathogen *Vibrio vulnificus* under the hypothesis that iron is one of the main signals triggering survival mechanisms both inside and outside its hosts. To this end, we selected a strain from the main zoonotic clonal-complex, obtained a mutant in the ferric-uptake-regulator (Fur), and analyzed their transcriptomic profiles in both iron-excess and iron-poor conditions by using a strain-specific microarray platform. Among the genes differentially expressed, we identified around 250 as putatively involved in virulence and survival-related mechanisms. Then, we designed and performed a series of *in vivo* and *in vitro* tests to find out if the processes highlighted by the microarray experiments were in fact under iron and/or Fur control. Our results support the hypothesis that iron acts as a niche marker, not always through Fur, for *V. vulnificus* controlling its entire life cycle. This ranges from survival in the marine environment, including motility and chemotaxis, to survival in the blood of their hosts, including host-specific mechanisms of resistance to innate immunity. These mechanisms allow the bacterium to multiply and persist inside and between their hosts.**

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## Introduction

*Vibrio vulnificus* is an emerging zoonotic pathogen that inhabits aquatic ecosystems from temperate, tropical and subtropical climates, in which it survives as planktonic form or associated with the mucosal surfaces of aquatic animals (Oliver, 2015). The species is subdivided in three biotypes (Bt) that differ in phenotypic traits and host range (Tison *et al.*, 1982; Bisharat *et al.*, 1999). The three Bts are considered opportunistic human pathogens while only Bt2 is also able to infect fishes (Tison *et al.*, 1982; Amaro and Biosca, 1996). This ability relies on a transferable virulence plasmid (called pVvbt2) that confers resistance to the fish innate immune response (Lee *et al.*, 2008; Valiente *et al.*, 2008). Among the Bt2-clones, the most dangerous for public health is the worldwide distributed clonal-complex formed by SerE strains (Sanjuán *et al.*, 2011). Human infection cases of known etiology related with this clonal-complex correspond to severe wound infections or secondary septicemia after diseased-eel handling (Amaro and Biosca, 1996; Sanjuán *et al.*, 2011; Haenen *et al.*, 2014).

Iron is an essential nutrient for *V. vulnificus*. In fact, the severity of the disease in humans is strongly related to iron levels in blood, being septicemic when these levels are abnormally high due to hemochromatosis or other disorders characterized by elevated iron levels (Horseman and Surani, 2011). Free-iron is presumably available to *V. vulnificus* in the nutrient-enriched environment characteristic of intensive fish-farming industry but it is unavailable inside the hosts due to nutritional immunity, an ancestral mechanism of defense common to fish and mammals (Weinberg, 2009). Thus, iron is present in host tissues either complexed to heme, forming part of the hemic proteins such as hemoglobin, or sequestered by transferrin in blood or lactoferrin in secretions, with all of these proteins binding $Fe^{3+}$ with an exceptionally high affinity (Hood and Skaar, 2012). *V. vulnificus* Bt2-SerE senses the lack of free-iron in blood and produces vulnibactin (a siderophore or iron-chelator) that competes with transferrin for iron together with its receptor VuuA, as well as a receptor for hemin. Both of these systems are required for virulence in humans and fish (Pajuelo *et al.*, 2014). However, Bt2-SerE can also produce a third iron-uptake system encoded by pVvbt2 that is eel-specific. This system relies on an outer membrane receptor for eel transferrin (Ftbp, fish transferrin binding

protein), which optimizes the growth of the pathogen in blood and makes it a highly virulent eel-pathogen ($LD_{50}$ for eels around 10–200 CFU per animal) (Amaro *et al.*, 1995; Pajuelo *et al.*, 2015).

The main iron-responsive transcriptional factor is Fur (ferric uptake regulator), most of the time acting as a negative regulator that uses iron as a cofactor (holo-repression) to control the transcription of iron acquisition-related genes (Hantke, 2001). However, recent studies suggest that Fur could also control other bacterial processes such as acid shock response, chemotaxis, metabolic pathways, bioluminescence, and production of toxins and other virulence factors, and, in some cases, through a positive regulation (Troxell and Hassan, 2013). In the case of *V. vulnificus*, it has been demonstrated that Fur controls its own transcription as well as that of a few virulence genes (including those for iron-uptake) and two master-regulator genes, *smcR* and *rpoS* (Lee *et al.*, 2003; Alice *et al.*, 2008; Kim *et al.*, 2013).

In this study, we aimed to analyze the global response of *V. vulnificus* to changes in iron levels by simulating the infection of a fish from tank water (bacteria "would sense" a decrease in iron concentration) and the infection of a "susceptible" human (high iron levels in blood) from a diseased-fish (bacteria "would sense" an increase in iron concentration) under the hypothesis that iron is a global signal for both virulence and survival gene switching. To this end, we selected a strain belonging to the zoonotic clonal-complex, whose genome has been sequenced and annotated, and obtained a *fur* deletion mutant (Δ*fur*) and the corresponding complemented strain (c*fur*). Then, we analyzed the transcriptomic profiles of the wild-type and the mutant strains growing in presence and absence of free-iron, and performed a series of confirmatory phenotypic *in vivo* and *in vitro* experiments also using the complemented strain. For the transcriptomic analysis we developed a custom microarray platform containing probes for all the predicted ORFs in the genome of the strain used in this study, the Bt2-SerE isolate CECT4999.

## Results

### *Virulence in mice and eels*

The role of iron and Fur in virulence was tested by comparing the virulence for normal and iron-overloaded animals of the wild-type (CECT4999), the mutant (Δ*fur*) and the complemented (c*fur*) strains. Iron-pretreatment increased by more than 2 log units the susceptibility to vibriosis of intraperitoneal (i.p.)-infected mice, regardless of the inoculated strain, whereas it did not affect virulence in eels (Table 1) (iron-overloaded eels were not i.p. infected due to the high virulence of the pathogen when using this route). In addition, *fur* deletion slightly reduced virulence for both mice and eels, regardless of the infection route (Table 1), and delayed internal organ colonization (Fig. 1).

### *Differentially expressed genes*

A total of 1229 genes (25.78% of the total genes measured) were differentially expressed by CECT4999 grown in iron-poor versus iron-rich media while 1712 genes (35.91% of the total genes) were differentially expressed by Δ*fur* versus wild-type strain (Supporting Information Tables S1 and S2). Among the genes that showed the highest change in expression level in iron limitation, we found a strong induction of those related with iron acquisition (siderophores and ferrous iron), cold shock, plasmid genes (*vep06*, *vep23* or transthyretin and *vep71*), and DNA sulfur modification (*dndE*), whereas genes belonging to the flagellar operon, chemotaxis or drug resistance were remarkably repressed (Supporting Information Table S1). Interestingly the pattern of the genes most strongly regulated by Fur was similar to that of iron restriction, including upregulated genes in the *fur* mutant for iron acquisition (siderophores, ferrous iron and heme) and chemotaxis, and downregulated genes such as those related to flagellum biosynthesis (Supporting Information Tables S1 and S2). It is worth highlighting that most genes encoding response to stress, outer membrane proteins, uptake of different nutrients and metabolic pathways were found to

**Table 1.** Effect of *fur* in cursive deletion and iron concentration on virulence for eels and mice in *V. vulnificus*.

| | **Virulence for**[a] | | | | |
|---|---|---|---|---|---|
| | Mice | | Eels | | |
| **Strains** | Normal | Iron-overloaded | Normal (i.p.) | Normal (immersion) | Iron-overloaded (immersion) |
| CECT4999 | $2.6 \times 10^4$ | $<5 \times 10^1$ | $1 \times 10^2$ | $1.2 \times 10^6$ | $7.6 \times 10^5$ |
| Δ*fur* | $9 \times 10^4$ | $1 \times 10^2$ | $3 \times 10^3$ | $7.2 \times 10^6$ | $1 \times 10^6$ |
| c*fur* | $2.0 \times 10^4$ | ND | $1.25 \times 10^2$ | $7.1 \times 10^6$ | ND |

[a]Virulence was determined as the lethal mean dosis ($LD_{50}$) for both normal and iron-overloaded animals and was expressed as colony forming units (CFU) per g (intraperitoneally infected [i.p.] animals) or ml (immersion infected eels [Amaro *et al.*, 1995]) according to Reed and Muench (1938). Each value represents the average of two independent experiments.

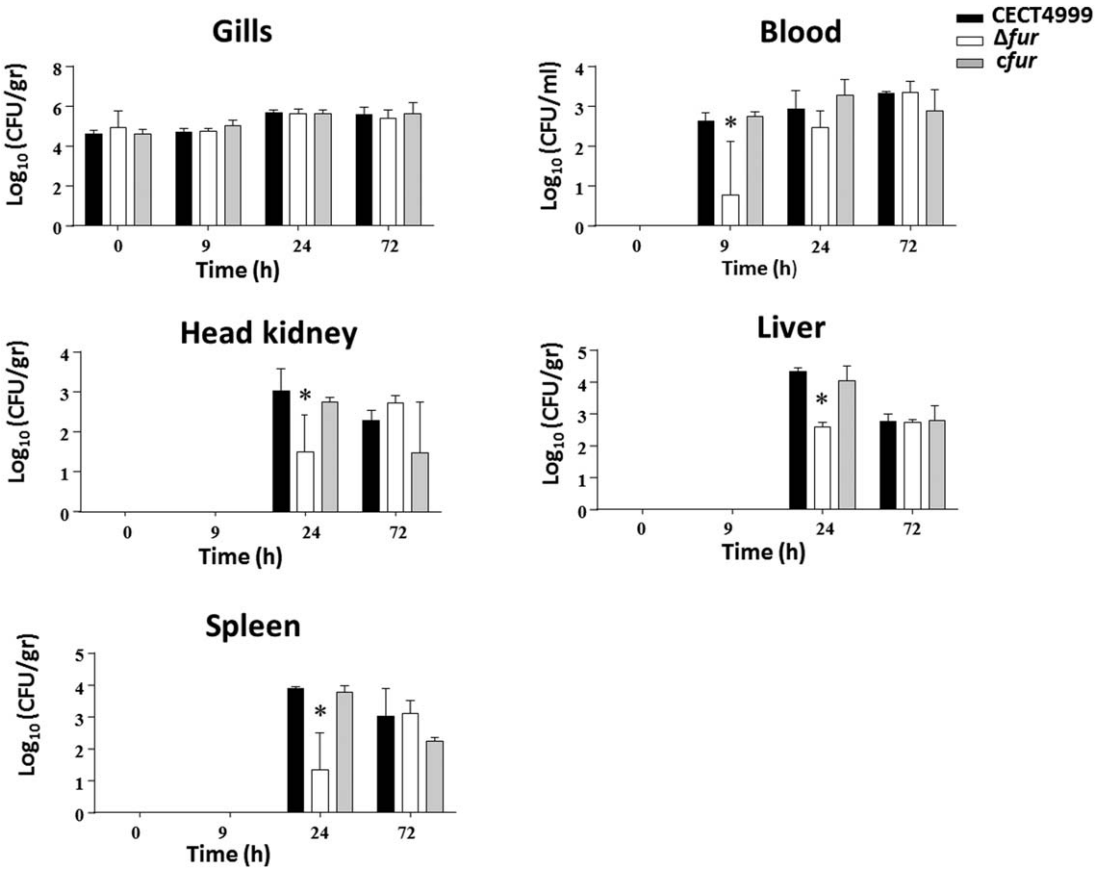


**Fig. 1.** Fur and *in vivo* colonization by *V. vulnificus* CECT4999. Eels were bath-infected with the wild-type strain (CECT4999), Δ*fur* and c*fur* at a dose of $10^6$ CFU/ml for 1 h. Then, bacterial colonization of external (gills) and internal (blood, liver, head kidney and spleen) organs was measured as bacterial counts (colony forming units or CFU) per g or ml at 0, 9, 24, and 72 h post-challenge and the bacterial counts per sampling point were statistically compared. Asterisks indicate significant differences in bacterial counts per sampling point (Student's *t*-test; $P < 0.05$).

be under putative control by Fur and iron (Supporting Information Tables S1 and S2). We found an acceptable correlation between microarray and qRT-PCR data corresponding to the 12 genes selected for validation (Supporting Information Table S3). Figures 2A and 3A show the distribution of the three regulation categories (up-, down- and nonregulation) per chromosome (Chr) and plasmid for putative iron- and Fur-controlled genes, respectively, and Supporting Information Fig. S1A the distribution of the eight mixed categories. Around 250 virulence- and survival-related genes were identified as putatively controlled by iron and/or Fur (Figs 2B and 3B, Supporting Information Tables S1 and S2), among them, genes encoding global transcriptional regulators (SmcR, the cAMP-mediated regulator, ToxR) and multiple genes that contain GGDEF and/or EAL domains (related with intracellular signaling) (Supporting Information Tables S1 and S2). The Fur protein was positively auto-regulated as previously published (Lee *et al*., 2007). Using the sequences of Fur boxes already described for *V. vulnificus* (Ahmad *et al*., 2009), we identified the putative Fur boxes for the virulence- and survival-related genes (Supporting Information Table S5). A consensus *V. vulnificus* Fur box motif built from putative Fur boxes founded is showed in Supporting Information Fig. S1B.

### *Iron and Fur in the life-cycle of* V. vulnificus

A series of phenotypic experiments were designed and performed to ascertain if the virulence- and survival-related processes identified by the transcriptomic study responded to exogenous iron levels and/or could be directly or indirectly controlled by Fur.

*Viable but non-culturable (VBNC) state. V. vulnificus* enters into the viable but non culturable (VBNC) state in seawater in cold months especially when temperatures are below 10°C (Whitesides and Oliver, 1997). To test whether entry into the VBNC state could be under iron and/or Fur control we analyzed the culturability of the three strains at 4°C under iron-excess or iron-poor conditions. Importantly,

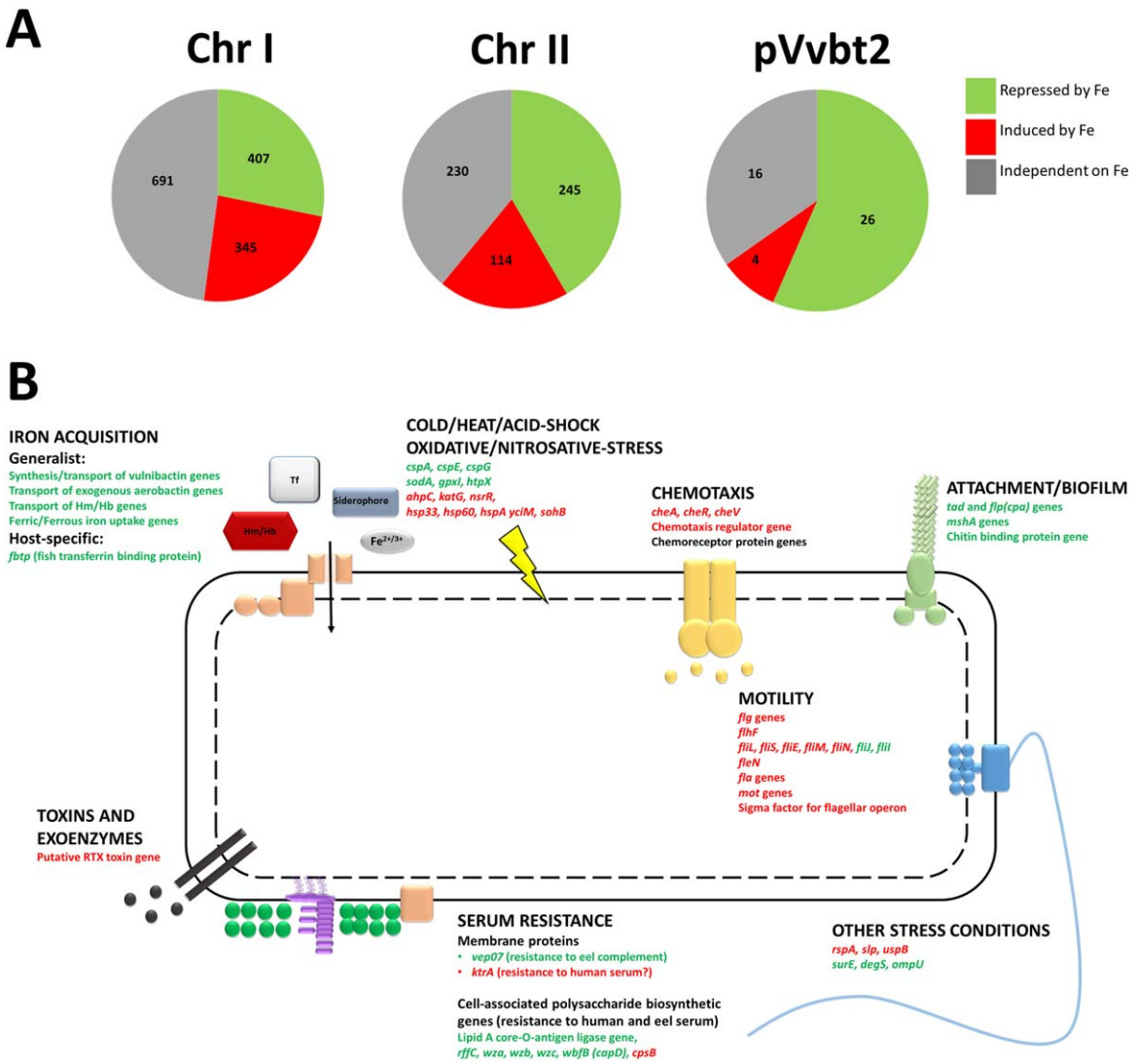


**Fig. 2.** Differentially expressed genes (DEGs) by *V. vulnificus* in response to iron (starvation versus excess). (A) Distribution of DEGs per regulation category and replicon (two chromosomes and one plasmid). The categories are represented in a color's code. (B) Main virulence- and survival-related processes that are putatively under control of iron. Red color: upregulated genes; Green color; downregulated genes: Black color: a group of related genes, some up- and other downregulated.

bacterial viability gradually decreased and the entrance into the VBNC state of Δ*fur* was accelerated (15 day before CECT4999), especially under iron-excess conditions (d10 in artificial seawater (ASW) (Mården *et al.*, 1988) with iron (ASW + Fe) versus d 21 in ASW without iron (ASW − Fe) (Fig. 4A). In contrast, the iron content of the medium did not affect the entry into the VBNC state of the wild-type strain (Fig. 4A). No differences were observed among the strains and conditions in resuscitation kinetics from the VBNC state (Fig. 4A). These results are compatible with the hypothesis that the entrance into the VBNC state in *V. vulnificus* would be directly or indirectly controlled by Fur in an iron-independent manner. Entry into the VBNC state in some vibrios has previously been suggested to be due to cold-induced loss of the antioxidative activity of catalase (KatG) and/or alkyl hydroperoxide reductase protein C (AhpC) (Oliver, 2010; Wang *et al.*, 2013; Rao *et al.*, 2014). Gene transcripts for both enzymes were significantly upregulated by iron (fold change values of 27.75 and 8.90 for *katG* and *ahpC*, respectively) and Fur (fold change values of 5.33 and 3.23 for *katG* and *ahpC* respectively). Our experimental assays confirmed that both genes were also upregulated in the wild-type strain after 3, 7, and 16 days post-inoculation at 4°C and that only *ahpC* expression was significantly less in the mutant in comparison to the wild-type strain (Fig. 4B). Interestingly, only *ahpC* has a putative

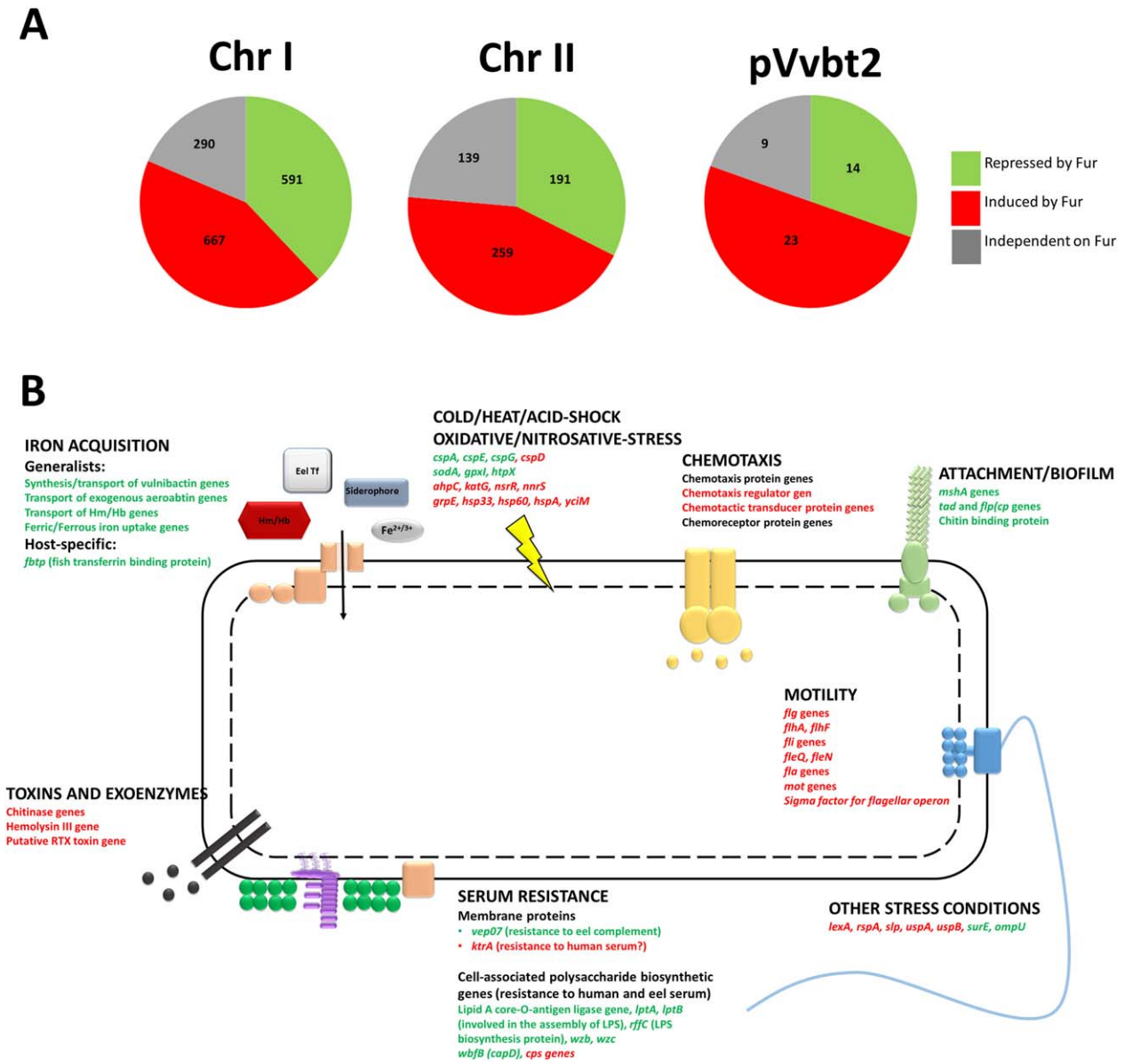


**Fig. 3.** Differentially expressed genes (DEGs) by *V. vulnificus* Δ*fur* versus wild-type. (A) Distribution of DEGs per regulation category and replicon (two chromosomes and one plasmid). The categories are represented in a color's code. (B) Main virulence- and survival-related processes directly or indirectly controlled by Fur. Red color: upregulated genes; Green color; downregulated genes: Black color: a group of related genes, some up- and other downregulated.

Fur box (Supporting Information Table S5) therefore being a candidate gene to be directly activated by Fur. Finally, the faster entrance into the VBNC state of Δ*fur* under iron-excess conditions could be explained by the formation of highly reactive hydroxyl radicals that have been reported to be produced under iron-excess conditions (Becker and Skaar, 2014) together with the increased sensitivity of the mutant to oxidative stress.

*Chemotaxis and motility.* Flagella and motility are known *V. vulnificus* virulence factors in mice (Lee *et al.*, 2004). The microarray results revealed that both iron and Fur could positively and negatively impact the transcription of genes involved in motility and chemotaxis (Figs 2B and 3B; Supporting Information Tables S1 and S2). In case of motility, almost all of the genes involved in flagellum biosynthesis together with different regulators and a specific sigma factor were revealed to be putatively upregulated by Fur and iron (Figs 2B and 3B; Supporting Information Tables S1 and S2). We then designed and performed a test confirming that motility corresponding to CECT4999 was significantly higher than that of Δ*fur* in all tested

 

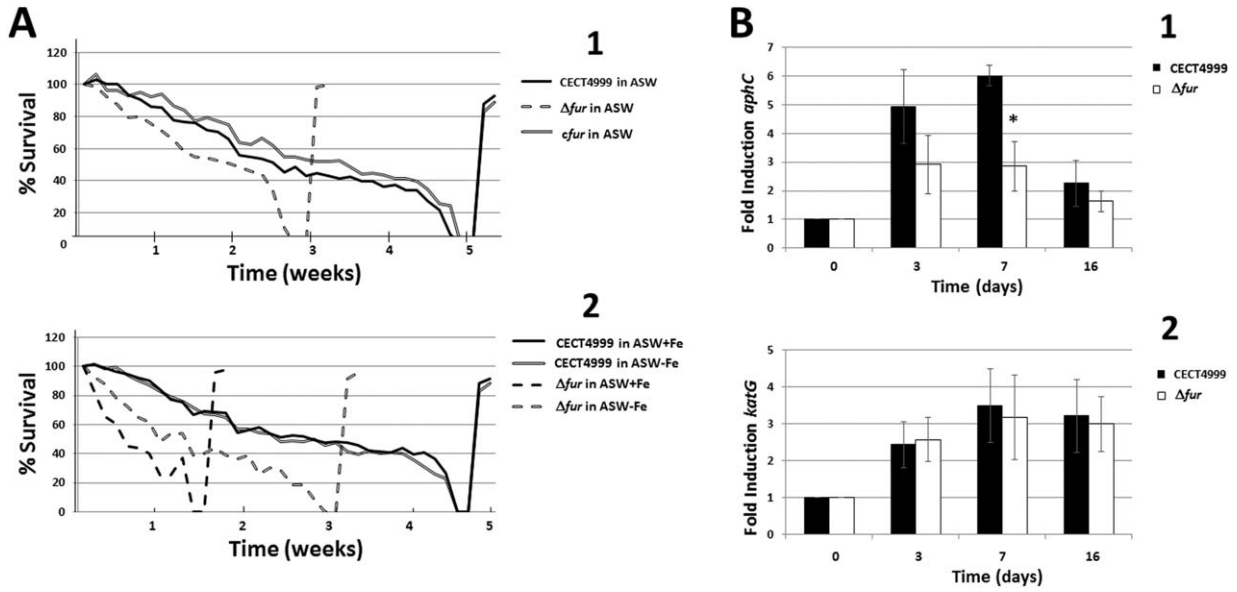


**Fig. 4.** Induction of VBNC state and resuscitation in *V. vulnificus* and transcription levels of *aphC* and *katG* during the induction of the VBNC state. (A) Cells from overnight cultures in MSWYE were transferred to ASW (1), ASW + Fe (2) or ASW-$Fe_{(D)}$ (2) and were maintained at 4°C with shaking (60 r.p.m.) till no bacteria was recovered on CM9A plates (VBNC state) and then the VBNC-cultures were placed at 22°C with shaking (60 r.p.m.) and resuscitation was monitored by plate counting on CM9A plates. Percent (%) survival refers to percentage of culturable bacteria (CFU/ml on CM9A) with respect to time 0 (100%). All values of % survival for Δ*fur* were significantly lower than those corresponding to CECT4999/c*fur* ($P < 0.05$) from day 2 till the induction of the VBNC state. (B) Transcription levels of *aphC* (1) and *katG* (2) determined as fold induction by qRT-PCR at 0, 3, 7 and 10 days of bacteria incubation in ASW at 4°C. *Significant differences ($P < 0.05$).

conditions. Furthermore motility of all of the strains, except that of the mutant, was enhanced significantly when iron was added (Fig. 5A). Unexpectedly, the motility of the mutant decreased significantly, as was the case for the rest of strains, when iron was removed from the growth medium (Fig. 5A). This indicates that other regulators, distinct from Fur could be involved in the motile response to variable iron conditions. The microarray results revealed that iron and Fur could also control the transcription of chemotaxis-related genes (Figs 2B and 3B; Supporting Information Tables S1 and S2). Since motile *Vibrio* cells are positively chemo-attracted by blood liberated by eel skin wounds (Valiente *et al.*, 2008), the chemotaxis toward eel and human plasma were tested under iron-excess and iron-poor conditions (Fig. 5B). Plasma and hemolytic plasma positively attracted CECT4999, and this attraction was significantly enhanced by iron while *fur* deletion significantly decreased chemoattraction across all plasma types (Fig. 5B). Interestingly, Δ*fur* was repelled by human plasma, which suggests a higher sensitivity to the bactericidal action of human serum. The flagellum- and chemotaxis-related genes with putative Fur boxes are indicated in Supporting Information Table S5. In conclusion, both chemotaxis toward blood and motility in *V. vulnificus* would be controlled directly or indirectly by Fur in an iron-dependent manner, although motility also seems to involve other iron-responsive regulator or regulators.

*Attachment and biofilm formation on abiotic surfaces. V. vulnificus* is able to adhere and form biofilm on biotic and abiotic surfaces (Paranjpye and Strom, 2005). Among the attachment genes putatively regulated by Fur and/or iron we identified some of those encoding a putative Tad (tight adherence) system (the machinery required for the assembly of an adhesive Flp [fimbrial low-molecular-weight protein] pilus) together with the *flp* (*cpa*) genes as well as those putatively encoding a MSHA pilus (Supporting Information Tables S1 and S2). Interestingly, *tad*, *flp (cpa)*, and *msha* genes were mostly downregulated by iron and Fur (Figs 2B and 3B; Supporting Information Tables S1 and S2) however none of these genes contains a putative Fur box. Biofilm production on a hydrophobic surface (polystyrene) was then analyzed and we found that this was time- and iron-dependent. Biofilms increased gradually, achieving a maximum at 1 week, and were decreased with increased iron concentration (Fig. 5C). These results suggest that biofilm formation and/or its dispersal is an iron-dependent process in which Fur does not play an essential role.

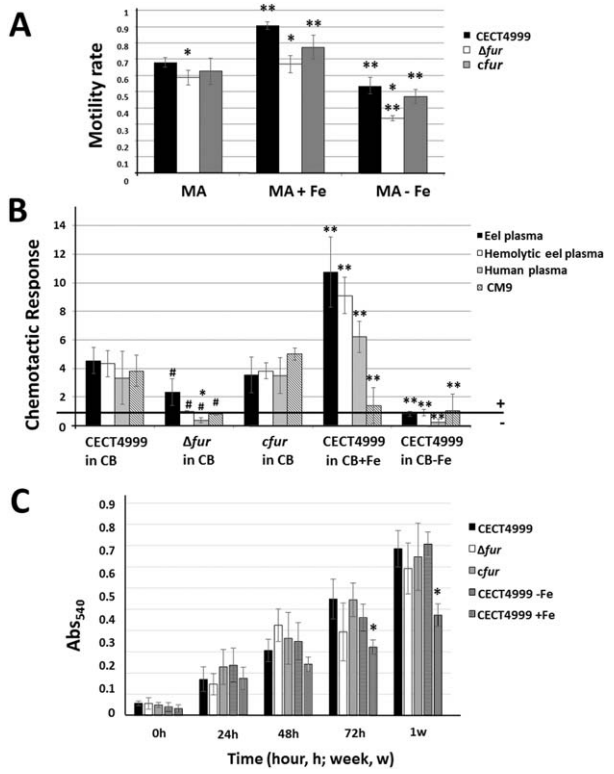


**Fig. 5.** Motility, chemotaxis and biofilm formation in *V. vulnificus*. (A) Motility on Motility agar (MA) measured as Motility rate (Mr) ("colony" surface in $cm^2$/log of "colony" bacterial number in CFU). *Significant differences in Mr between strains (Δ*fur* or c*fur* versus CECT4999) ($P < 0.05$); **significant differences between conditions (with iron [MA + Fe] or without iron [MA-$Fe_{(D)}$] versus MA) for the same strain ($P < 0.05$). (B) Chemotaxis toward eel plasma (EP), hemolytic eel plasma (HEP), human plasma (HP), and CM9 was measured as Chemotactic response (Cr) (ratio bacterial numbers in EP-HEP-, HP-, or CM9-capillaries versus control-capillaries [containing Chemotaxis buffer (CB)]). Horizontal line marks the borderline between positive and negative chemotaxis. *Significant differences in Cr toward EP, HEP, or HP versus CM9 per strain ($P < 0.05$); #, significant differences between strains (Δ*fur* or c*fur* versus CECT4999) per tested attractant (EP, HEP, HP, CM9) ($P < 0.05$); **significant differences in function of iron content in the chemotaxis buffer (CB + Fe or CB-Fe versus CB) per tested medium for the strain CECT4999 ($P < 0.05$). (C) Biofilm quantification: bacteria were grown in polystyrene wells, planktonic bacteria were eliminated and biofilm was quantified after staining with crystal violet by measuring absorbance at 540 nm at 0, 24, 48, 72 h and 1 week post-incubation. *Significant differences between conditions (+Fe versus -Fe) for strain CECT4999 ($P < 0.05$).

*Toxins and exoenzymes.* A few genes for toxins (putative hemolysins) and proteases were identified as likely to be under iron and/or Fur control (Supporting Information Tables S1 and S2). None of them corresponded to the major toxins (Multifunctional Autoprocessive Repeat in Toxin, RtxA1 and the hemolysin VvhA) and exoenzymes (protease VvpE) of *V. vulnificus* (Shao and Hor, 2000; Lee *et al.*, 2004; 2013). We did not find significant differences in either cell-associated- or extracellular-hemolytic/proteolytic activities among strains and conditions (iron excess versus iron deficiency) (Supporting Information Fig. S2 and data not shown).

*Resistance to stress conditions.* A series of genes related to resistance to various stressors from the innate immune response were identified to be putatively controlled by iron and/or Fur (Figs 2B and 3B; Supporting Information Tables S1 and S2). Firstly, we tested resistance to the antimicrobial compounds polymyxin B, lysozyme, and deoxycholate (a bile salt) and found that the MIC (minimal inhibitory concentration) values did not change with either iron concentration or *fur*-deletion (MICs: polymyxin B, 500 U/ml; lysozyme, 500 μg/μl; human apotransferrin, 50 μM). Bacterial survival for all experimental strains was determined under acidic or oxidative conditions, as well as the effect of nitric oxide (NO) on growth in both strains (Fig. 6). Survival decreased significantly in all strains when the pH of the medium was adjusted to 5 instead of 7 (Fig. 6A). Univariate analysis of the variance showed that all single variables, strain (Δ*fur* versus CECT4999), iron (MSWYE (Marine Seawater Yeast Extract [Biosca and Amaro, 1996] +Fe versus MSWYE − $Fe_{(Tf)}$) and pH (acid versus neutral) were highly significant at 4 and 6 h post incubation (Supporting Information Table S4). At these times all interactions with the exception of strain*pH were also highly significant (Supporting Information Table S4). When the effect of the pH*iron on the growth of each single strain was analyzed separately we uncovered a strongly significant effect only for the mutant ($P < 0.0001$) (data not shown). These results support the observation that the mutant's survival at pH 5 was strongly dependent on iron where it was not recoverable after 4 h of incubation in MSWYE + Fe. In contrast the mutant survived significantly more than the wild-type strain in MSWYE-$Fe_{(Tf)}$ (supplemented with human apotransferrin) (Fig. 6A; times 2 and 4 h). The deleterious effect of acid pH on the mutant in presence of an iron excess could be due to the spontaneous production of radical peroxide ions previously reported to be induced under these conditions (Teranishi *et al.*, 2016). In fact, the mutant was significantly more sensitive than the wild-type strain to $H_2O_2$ (Fig. 6B) where the observed sensitivity was independent on iron levels at pH 7 (Fig. 6B) and strongly dependent at pH 5 (no cultivable cell was recovered on agar plates) (data not shown). In the case of NO, the mutant was significantly more resistant than the wild-type strain both under iron-excess (time 4 h) and iron-starvation conditions (times 4 and 7) (Fig. 6C). Univariate analysis highlighted that all single variables, strain (Δ*fur* versus CECT4999), iron (MSWYE + Fe versus MSWYE − $Fe_{(Tf)}$) and NO (with versus without) were highly significant (Supporting Information Table S4; times 4 and

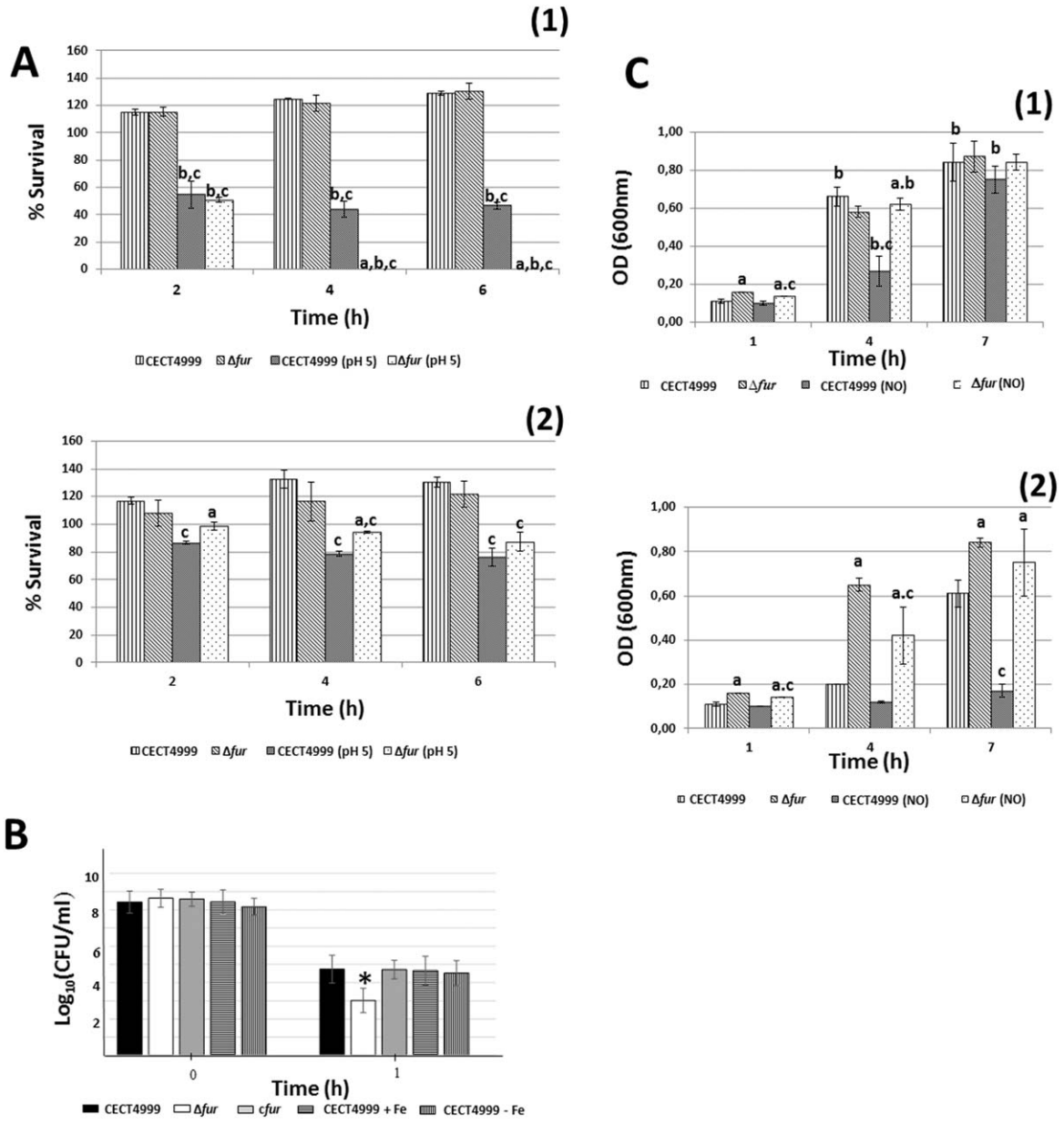


**Fig. 6.** Sensitivity to acid-, oxidative- and nitrosative-stress in *V. vulnificus*. Resistance to acid stress (A) was tested by incubating bacteria in MSWYE + Fe (A1) or MSWYE-$Fe_{(Tf)}$ (A2) adjusted to neutral (7) or acid pH (5), and counting survivors (% survival) on agar plates at different 2-h intervals for 8 h. a, significant differences between strains (Δ*fur* versus CECT4999) for the same culture medium and pH condition (normal or acid medium); b, significant differences between culture media (MSWYE + Fe versus MSWYE − $Fe_{(Tf)}$) for the same strain and pH condition; c, significant differences between pH conditions (acid versus neutral medium) for the same strain and culture medium ($P < 0.05$). Resistance to $H_2O_2$ (B) was tested by incubating bacteria in PBS-$H_2O_2$ (0.1%), respectively, and counting survivors (CFU/ml) on agar plates at different time intervals. *Significant differences between strains (Δ*fur* or *cfur* versus wild-type) ($P < 0.05$). Resistance to nitrosative stress (C) was tested by following growth in CM9 + Fe (C1) or CM9 − $Fe_{(Tf)}$ (C2) with and without nitric oxide (NO) 400 μM (NO was added as dipropylenetriamine NONOate [DPTA NONOate is a NO donor] from Cayman chemicals) by measuring absorbance at 600 nm at 1-h intervals for 7 h. a, significant differences between strains (Δ*fur* versus CECT4999) for the same culture medium and NO condition (with or without NO); b, significant differences between culture media (CM9 + Fe versus CM9 − Fe) for the same strain and NO condition; c, significant differences between NO conditions (with NO versus without NO) for the same strain and culture medium ($P < 0.05$).

7 h post incubation). However only the interaction strain*iron was highly significant at both sampling times (Supporting Information Table S4). When the effect of the three variables on the growth of each single strain was separately analyzed it was revealed that the interaction iron*NO was strongly significant only for the wild-type strain ($P < 0.0001$). These results support the finding that the wild-type strain's resistance to NO was strongly

dependent on iron (Fig. 6C). The main mechanism by which NO inhibits bacterial growth is by binding iron and forming dinitrosyl iron complexes bound to iron–sulfur bacterial proteins, inhibiting their functions (Toledo *et al*., 2008). In a medium such as CM9 (M9 minimal medium broth supplemented with casaminoacids 0.2%, wt/vol [Miller, 1972]) with transferrin CM9 − $Fe_{(Tf)}$ with practically no free-iron, all NO would bind to bacterial proteins and the bactericidal effect would be stronger than in an iron-rich medium thus explaining the higher sensitivity to NO of the wild-type strain. All these measures provide a functional validation of the data obtained from the microarray analysis where *sodA* that encodes an enzyme involved in resistance to acid-stress in *V. vulnificus* (Kim *et al*., 2005), *nsrR*, encoding a repressor specifically dedicated to sensing nitric oxide (Bodenmiller and Spiro, 2006), and *ahpC*/katG (related to entry into the VBNC state) were up-, down- and downregulated, respectively, in the mutant. As previously mentioned for *ahpC*, *sodA* genes also contain a Fur box (Supporting Information Table S5), while no Fur box was detected in *nsrR*. In conclusion, resistance to oxidative-stress could be directly controlled by Fur in an iron-independent manner; resistance to acid-stress also by Fur but in an iron-dependent way and, finally, resistance to nitrosative stress should be a process indirectly depending on Fur.

*Growth under iron-restriction.* As expected, genes involved in iron acquisition were putatively downregulated by Fur and iron with very few exceptions (Figs 2B and 3B, and Supporting Information Tables S1 and S2). Amongst them, the plasmid gene *vep20* encoding a recently described eel-transferrin receptor, Ftbp, is worth highlighting due to its key role in eel virulence (Pajuelo *et al*., 2015). The genes containing putative Fur boxes are indicated in Supporting Information Table S5. When all the strains were grown with hemin or holotransferrin as the sole iron source Δ*fur* grew faster achieving significantly higher absorbance values than the wild-type and the complemented strains during the log phase of growth (Fig. 7). These data again validate the results obtained using the microarray therefore highlighted the validity and value of this approach.

*Resistance to serum.* A series of genes related to resistance to the bactericidal action of fresh serum were identified. The plasmid gene, *vep07*, involved in resistance to eel serum (Lee and Hor, 2010) and *trkH*, a gene related to a potassium-uptake system involved in human serum resistance (Chen *et al*., 2004), were found to be differentially expressed depending upon the medium iron content and/or *fur* deletion (Supporting Information Tables S1 and S2). *vep07* was upregulated both under iron restriction and in the mutant while *tkrH* was upregulated in the mutant, independently of iron, and showed a putative Fur box (Figs 2B and 3B, Supporting Information Tables S1, S2, and S4). Interestingly, a second potassium uptake pump

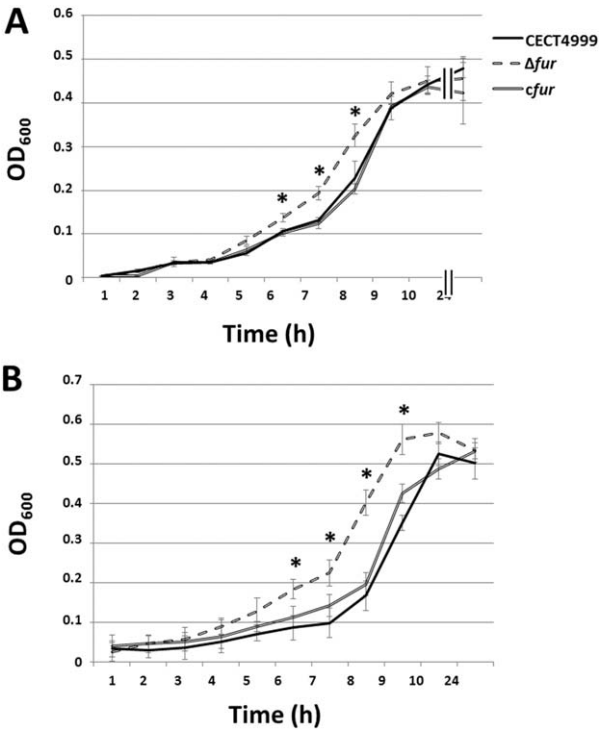


**Fig. 7.** Bacterial growth in presence of hemin and transferrin as the sole iron sources. The three strains of *V. vulnificus* were inoculated in CM9-Hm (A) and CM9 − $Fe_{(Tf)}$ (B) and growth was followed by measuring the $OD_{600}$ at regular time intervals. *Significant differences in values of $OD_{600}$ between the Δ*fur* versus wild-type/c*fur* strains ($P < 0.05$).

(depending on *ktrAB* locus), found in *V. vulnificus* by Chen *et al*. (2004), but still uncharacterized, was found to be upregulated by iron, independently of Fur (Figs 2B and 3B, and Supporting Information Tables S1 and S2). This finding is compatible with the hypothesis that the second system would be inducible in iron-overloaded human serum. We also found genes involved in Lipid A-core, O-antigen, capsular polysaccharide and EPS (extracellular polysaccharide) biosynthesis as differentially expressed under the assayed conditions (Figs 2A and 3A and Supporting Information Tables S1 and S2). Capsular polysaccharides have been previously shown to provide resistance to the bactericidal effect of human serum but not with resistance to eel serum (Wright *et al*., 1990; Biosca *et al*., 1993). Most of the genes involved in core-lipid A and antigen O biosynthesis were putatively repressed by Fur, with or without iron dependence, whereas those involved in capsule/EPS biosynthesis were mostly upregulated by Fur in an iron-dependent manner (Supporting Information Tables S1 and S2). In agreement, we found evidence of outer-membrane alterations between the wild-type and the mutant revealed by differences in saponin sensitivity, a non-ionic surfactant of vegetal origin (Fig. 8A). From all DEGs related to polysaccharide

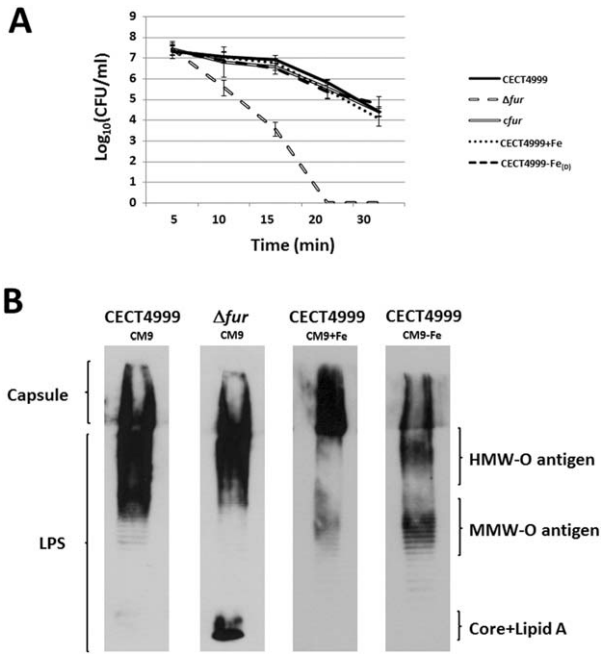


**Fig. 8.** Cell-associated polysaccharides of *V. vulnificus*. (A) The sensitivity to detergents was tested by incubating bacteria in PBS-saponin (100 μg/ml) for 30 min at 28°C and drop-plate counting at specific sampling points. (B) Cell-associated polysaccharides (LPS + capsule) were extracted with the method of Hitchcock and Brown (1983), separated by SDS-PAGE and immunostained with rabbit primary antibody anti-CECT4999 and secondary anti-rabbit HRP-conjugated. Lane A, LPS + capsule from wild-type strain grown in CM9; Lane B, LPS + capsule from Δ*fur* grown in CM9; Lane C, LPS + capsule from wild-type strain grown in CM9 + Fe; Lane D LPS + capsule from wild-type strain grown in CM9-$Fe_{(Tf)}$. HMW, high molecular weight; MMW, medium molecular weight.

biosynthesis, only three genes involved in core-lipid A biosynthesis contain a putative Fur box (Supporting Information Table S5). The cell-associated polysaccharides from the wild-type, the mutant and the complemented strains, grown in presence or absence of iron, were extracted and the polysaccharides quantified and analyzed. As predicted by microarray analyses of nascent bacterial transcripts, the quantity of polysaccharides (μg per $10^8$ cells) significantly increased with iron concentration in the wild-type ($P < 0.05$), significantly decreased in Δ*fur* with respect to the wild-type/complemented strain ($P < 0.05$) and did not significantly change in the *fur* mutant with iron ($P > 0.05$) (Table 2). Finally, the cell-associated polysaccharide pattern varied among strains and conditions: i.e. the core-lipid A increased with *fur* deletion while the capsule increased concomitantly with a decrease in O-antigen when the wild-type strain was incubated under iron-excess conditions (Fig. 8B).

## Discussion

The results obtained in this study support the hypothesis that iron has a key role in controlling virulence and survival in the aquatic environment of the pathogen *V. vulnificus*. The custom-built microarray platform generated from all predicted ORFs in the Bt2-SerE isolate CECT4999 highlighted that iron stimulon involves 25% of the genes present in the genome. This perhaps is not surprising given that *V. vulnificus* is a septicemic microorganism that has the ability to grow and survive in the blood, a medium where free-iron is bound by transferrin. In a similar manner ~20% of the gonococcal genome is regulated in response to growth under iron-replete versus -depleted conditions both dependently and independently of Fur (Ducey *et al*., 2005; Jackson *et al*., 2010). Furthermore most of the genes in the plasmid, pVvbt2, were repressed by iron which would ensure its expression in eel blood. This plasmid encodes a host-specific resistance system to the bacteriolytic and bacteriostatic effect of eel serum that is essential for virulence (Lee *et al*., 2008). We also uncovered evidence that Fur could repress as well as activate genes both independently and dependently of iron. These results suggest that the Fur protein in *V. vulnificus* would be as versatile as Fur in *V. cholerae* (Mey *et al*., 2005). Several mechanisms have been suggested to explain how Fur acts as an activator, with the "antirepressor" model as the major mechanism for Fur-dependent activation (Troxell and Hassan, 2013). In this study we found that Fur directly or indirectly represses the transcription of *smcR*, *crp*, and *toxR*, thus directly affecting processes controlled by these global regulators. Thus, the repression of *smcR* by Fur could for example "activate" the transcription of genes repressed by this global regulator. Although the repression of *smcR* by Fur has already been demonstrated (Kim *et al*. 2013) this is the first time to our knowledge that the effect

**Table 2.** Effect of iron concentration and *fur* deletion on the quantity of cellular-associated polysaccharydes.

| | CECT4999 | | | Δ*fur* | | |
|---|---|---|---|---|---|---|
| | CM9 − Fe | CM9 | CM9 + Fe | CM9 − Fe | CM9 | CM9 + Fe |
| μg/$10^8$ cells | 219.84 ± 9.7≠ | 235.2 ± 6.8** | 298.84 ± 18.3 | 178.6 ± 22* | 187.1 ± 3.6* | 196.9 ± 10.8* |

*Significant differences between strains (Δfur versus CECT4999) for the same condition ($P < 0.05$).
**Significant differences between conditions (CM9 versus CM9 + Fe) for the same strain ($P < 0.05$).
≠Significant differences between conditions (CM9 − Fe versus CM9 + Fe) for the same strain ($P < 0.05$).

of Fur on ToxR and Crp expression has been reported in *Vibrio*.

Figure 9 represents a hypothetical life cycle for *V. vulnificus* based on the main results obtained in the present study. *V. vulnificus* is a marine pathogen from warm brackish-water ecosystems that can infect fish and humans causing septicemia (Jones and Oliver, 2009). Previous studies performed in artificial and natural seawater microcosms demonstrated that *V. vulnificus* can survive under nutrient starvation in seawater for years maintaining its pathogenic potential for fish and humans by entering into the VBNC state (Marco-Noales *et al*., 1999). This state can be induced in the laboratory by incubating the pathogen at 4°C (Wolf and Oliver, 1992; Biosca *et al*., 1996; Whitesides and Oliver, 1997). Our results clearly show that Fur is involved in the entrance into the VBNC state as the Fur mutant becomes non-culturable much earlier than the wild-type strain. We also found experimental evidence relating this process to a reduction in oxidative stress resistance (experiments of survival in $H_2O_2$) concomitantly with a downregulation of *aphC* expression, a gene that possesses a Fur box (Fig. 9). Collectively these results support the hypothesis of Li *et al*. (2014) about the role of resistance to oxidative stress in the entrance into the VBNC state.

*V. vulnificus* resuscitates from the VBNC state with an increase in temperature in the presence of nutrients (Marco-Noales *et al*., 1999). Our study suggests that neither iron nor Fur have a clear role in the resuscitation from the VBNC state, at least under our experimental conditions (Fig. 9). Although nutrients are scarce in the open sea, they are more abundant in the coast regions, especially in estuarine waters, a habitat common for both *V. vulnificus* (Oliver, 2015) and the eel (Aarestrup *et al*., 2009). We have found clear evidence that, once resuscitated, *V. vulnificus* could be attracted by blood liberated from a human's or an eel's wound; attraction and bacterial motility being favored in an iron-rich environment (Fig. 9). Furthermore it is likely that both motility and chemotaxis to blood are probably activated by Fur and here we highlight possible candidate genes that may be directly involved. Previous studies have demonstrated that flagellum biosynthesis in *V. vulnificus* is controlled by direct binding of *smcR* to the promoter of *flhF*, a gene driving activation of flagellum biosynthesis (Kim *et al*., 2012). In this study *smcR* was upregulated in the *fur* mutant, suggesting an additional indirect activation of flagellum biosynthetic genes by Fur. The regulatory role of Fur in flagellum biosynthesis remains to be investigated in further studies. On the other hand, the genes for Flp (Cpa) and MSHA pili biosynthesis were mostly upregulated under iron-restriction and in the *fur* mutant, although in this case we did not find related Fur boxes. Thus, the bacterium would express the flagellum being motile in iron-excess and could express a Flp (Cpa)/MSHA pilus remaining sessile under iron-restriction (Fig. 9). In any case, to our knowledge this is the first time that Flp (Cpa)/MSHA pilus and flagellum biosynthesis have been linked with iron and Fur in *Vibrio*. Flp (Cpa) and MSHA pili have a role in attachment to surfaces in *Pseudomonas* and *V. cholerae* (Tomich *et al*., 2007; Utada *et al*., 2014). Thus we analyzed biofilm formation in iron-excess and iron-starvation by the wild-type and mutant strains. Our results suggest that iron starvation could directly or indirectly activate biofilm formation in *V. vulnificus* as biofilm production by the wild-type strain was decreased in iron-excess (Fig. 9). In parallel, under iron-restriction capsule production decreased in direct agreement with the reported inverse correlation between capsule production and biofilm production in *V. vulnificus* (Joseph and Wright, 2004). Iron is presumably sequestered by eel lactoferrin thus biofilm formation on the external and internal mucous surfaces of the eel would be favored. Our hypothesis is that all *V. vulnificus* Bts are able to colonize eel or fish surfaces by forming biofilms.

*V. vulnificus* either as free living form or associated with particulate material could be accumulated by oysters, the main reservoir for this pathogen and, then cause human infection after ingestion (Oliver, 2015). After colonizing a fish/human wound after contact, or human intestine after ingestion, *V. vulnificus* could invade bloodstream where it would sense a reduction in iron concentration due to iron being bound to transferrin (Fig. 9). The pathogen is then able to sequester iron from hemin/hemoglobin (main receptor HupA), vulnibactin, (receptor VuuA) and eel transferrin, (receptor Ftbp) (Pajuelo *et al*., 2014; 2015). In this study, we found that virtually all genes involved in the three iron-acquisition systems were repressed by iron and Fur, which would ensure that the bacterium, once in blood, would activate the transcription of genes involved in iron uptake to multiply (Fig. 9). However, the bacterium also needs to resist the bactericidal action of complement to achieve efficient multiplication in this environment. The capsule of *V. vulnificus* is the only virulence factor unequivocally involved in resistance to human serum (Wright *et al*., 1990). As previously mentioned we found both phenotypic and transcriptomic evidence that iron restriction through Fur would produce a decrease in the amount of capsular polysaccharide (Fig. 9). The fact that the capsule could be repressed in normal human blood could explain why most of *V. vulnificus* strains preferentially cause sepsis in patients with high iron-levels in blood, regardless of the infection route (oral or contact) (Oliver, 2015). On the contrary, the ability to resist eel complement is attributable to the plasmid gene *vep07* but not to the capsule (Amaro *et al*., 2015; Biosca *et al*., 1993). The gene, *vep07* containing a putative Fur box, was strongly activated under iron restriction (Fig. 9). The mechanism of action of Vep07 is unknown, but the deletion of the gene results in sensitivity to eel complement but not to human complement (Lee *et al*., 2008). We also found evidence that Fur directly or

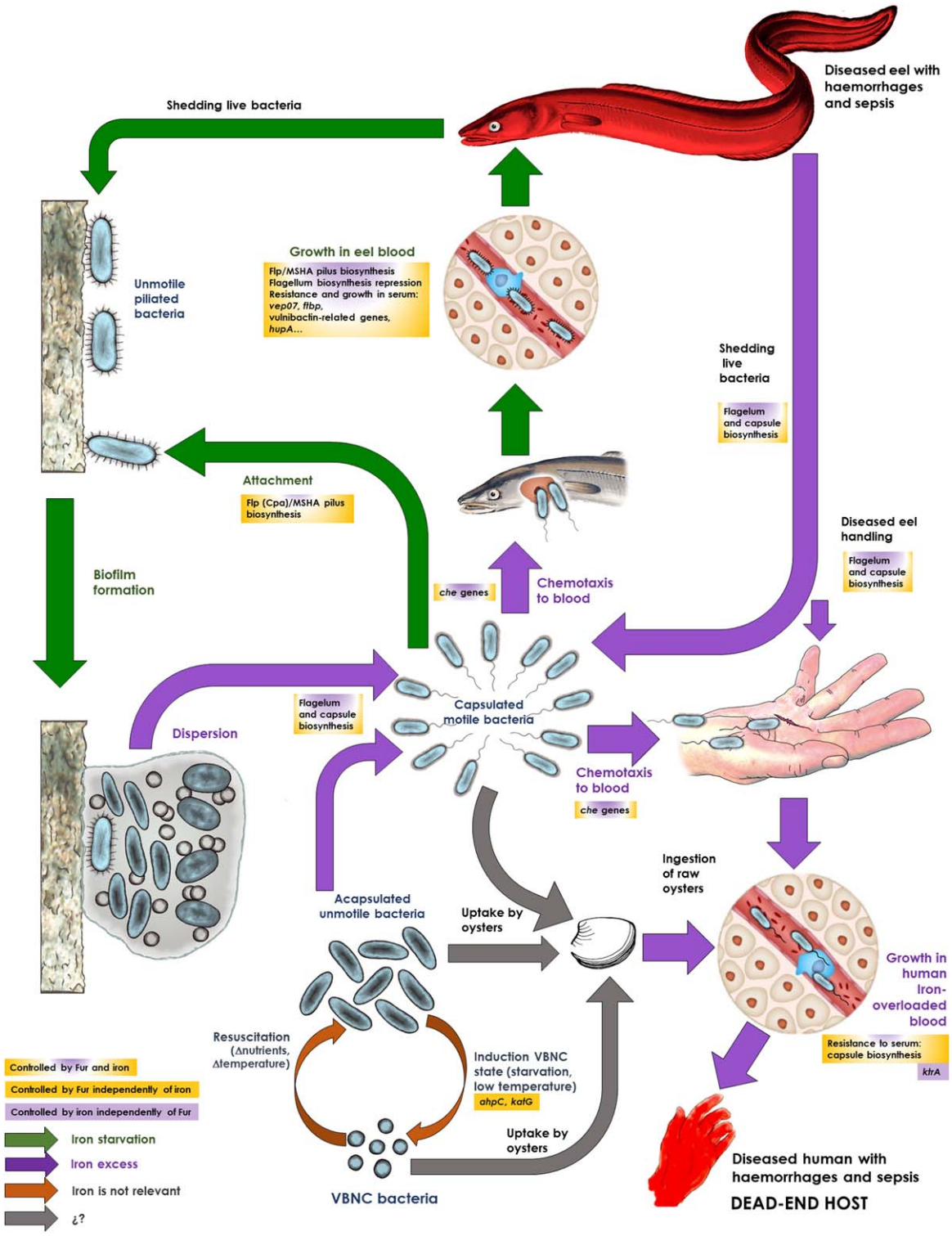


**Fig. 9.** Iron and Fur and the life cycle of the zoonotic pathogen *V. vulnificus*. This figure summarizes the role of iron and Fur in the life cycle of *V. vulnificus*. Only the strains that possess the virulence plasmid pVvbt2 (Bt2) could invade successfully the eel blood and cause death by septicemia. The rest of the strains or Bts would colonize mucosal surfaces of the eel or other fish species without invading them.

indirectly represses genes for lipid A-core biosynthesis. Thus, the mutant presented an altered OM pattern together with a notable increase in sensitivity to detergents such as saponin, in both cases without a clear relationship to the iron concentration. Furthermore we identified a Fur box in three of the genes involved in core-lipid A biosynthesis, which would be candidates to be directly regulated by Fur. This would be the first report in which a link between iron, capsule-LPS biosynthesis and Fur in *Vibrio* is suggested. Our results also suggest that Fur may play a role in resistance to more general stressors related to innate immunity (NO, oxidative stress, acid pH…) in an iron-independent way. Thus, Fur would activate resistance to oxidative stress and would repress resistance to acid and nitrosative stress, in both cases directly or indirectly.

The virulence assays performed in eels and mice revealed that the *fur* mutant showed a slight increase in $LD_{50}$ for both species together with a delayed colonization of internal organs, which contrasts with the reduction in virulence of more than 2 log units reported for other pathogens in different animal models including fishes (Porcheron and Dozois, 2015). Remarkably, the effect of iron in virulence was much more evident because virulence for mice and eels increased in more than 2 log. units with iron-pretreatment, which is in accordance with previous reports for *V. vulnificus* (Amaro *et al*., 1994). Thus, the minor variation in virulence of the *fur* mutant would be the result of the sum of different processes: the faster growth in iron-restricted conditions together with the higher resistance to acid and nitrosative stresses contrasts with the detrimental changes to the OM and a higher sensitivity to oxidative stress.

Human vibriosis differs from fish vibriosis in that the most severe form of the disease in humans (sepsis) is mostly produced when iron levels in blood are high while this condition is not necessary in the eel. In the present study we have found evidence that iron concentration in the animal's blood determines the outcome of human and animal disease. Thus, the higher virulence toward eels would be partially due to the two plasmid-encoded proteins that confer specific resistance to the bactericidal (Vep07) and bacteriostatic effect (Ftbp) of eel blood. Both targets are repressed by Fur and iron therefore are expressed in eel blood under normal circumstances. In contrast, this pathogen only possesses generalist systems to overcome the bacteriostatic and bactericidal action of human serum, the first ones those depending upon HupR and VuuA are repressed by Fur and iron, and would be expressed in normal human serum but the second ones those depending upon capsule biosynthesis would only be optimally expressed in iron-overloaded serum, thus explaining why human sepsis is correlated to iron-overloading.

In summary our results support the hypothesis that iron, not always through Fur, is one of the main signals acting as niche marker for *V. vulnificus*. Iron impacts the entire life cycle of the pathogen from its survival in the marine environment, including motility and chemotaxis, to its survival in the blood of their hosts. In blood, iron concentration would be the key signal for this septicemic bacterium, triggering the expression of genes involved in survival and resistance to the innate immune response (the plasmid genes *vep07* and *ftbp* in eel's blood and the chromosomal genes involved in capsule biosynthesis in iron-overloaded human blood) allowing the bacterium to multiply and persist inside their hosts.

## Experimental procedures

### *Bacterial strains, growth media and conditions*

The bacterial strains used in the study are listed in Table 3. The bacteria were routinely grown in LB-1/LBA-1 (Luria-Bertani broth/agar, 1% NaCl) or CM9/CM9A. If necessary, ampicillin (100 μg/ml), chloramphenicol (20 μg/ml for *Escherichia coli* and 2 μg/ml for *V. vulnificus*) or polymyxin B (50 U/ml) were added to the media. To analyze the effect of different

**Table 3.** 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 |

iron sources on growth, bacteria were grown in CM9 − $Fe_{(D)}$ (CM9 plus 50 μM 4,4 − Dipyridyl [Sigma-Aldrich, Madrid, Spain] ), CM9-Hm (CM9 − $Fe_{(D)}$ plus 0.1 μM bovine hemin [Sigma-Aldrich]), CM9 + Fe (CM9 plus 100 μM $FeCl_3$) and CM9 − $Fe_{(Tf)}$ (CM9 plus 40 μM of unsaturated human transferrin or apotransferrin, [Sigma-Aldrich]). *V. vulnificus* strains were incubated at 28°C and *E. coli* strains at 37°C for 18–24 h. All the strains were stored in LB-1 plus glycerol (17%) at −80°C.

### *DNA purification, PCR and generation of mutant and complemented strains*

The genomic DNA was extracted as described by Ausubel *et al*. (1999). DNA amplification by PCR was performed as described by Pajuelo *et al.* (2014) and primers were designed from the genome of *V. vulnificus* CECT4999 (NCBI Accession No. CP01436 for chromosome I, CP01437 for chromosome II and CP01438) and the virulence plasmid pR99 (AM293858). To obtain a Fur-deficient strain, *fur* deletion mutant was obtained by chitin-based natural transformation (Gulig *et al*., 2009) with slight modifications. Briefly, the regions of the chromosome corresponding to up-(1382 nt) and downstream (1329 nt) of *fur* gene were amplified using primer sets (Fur-1/Fur-2 and Fur-3/Fur-4) (Supporting Information Table S5) and cloned into the pGEMT-easy. Chloramphenicol resistance marker was inserted at *Xba*I site of the cloned construction thus obtaining plasmid pΔ*fur*. Plasmid was linearized by *Xmn*I digestion and introduced into *V. vulnificus* CECT4999 by natural transformation as previously described (Meibom *et al*., 2005). Transformants were selected using LB plates supplemented with chloramphenicol. To generate the complemented strain, c*fur*, the entire *fur* gene and its promoter region were 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*) was introduced into Δ*fur* strain by conjugation.

### *Microarray analysis*

*Microarray design.* The CECT4999-specific gene expression microarray (8 × 15 K) slides were custom designed with eArray software (Agilent technologies), following MIAME guidelines for array design (Brazma *et al*., 2001), taking as reference the predicted annotated ORF's of CECT4999 strain genome for the probes design. The arrays contained in total 4553 probes of 60-oligonucleotide length. These probes were distributed in 3 probes per target (13659) with an e-value of 0.0 and the rest were filled with internal control probes of Agilent. The platform was submitted to the GEO repository with the accession number GPL19040.

*Sample preparation, labeling and hybridization.* Total RNA from mid-log phase cultures was extracted with TRI reagent (Sigma). RNA was subjected to a DNase treatment with the TURBO™ DNase (Ambion, Madrid, Spain) and cleaned with the RNeasy® MinEute® Cleanup Kit (Qiagen, Barcelona, Spain) as described by the manufacturers. RNA concentration and integrity were measured by 2100 Bioanalizer (Agilent, Madrid, Spain). 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). To obtain cDNA, 200 μg of total 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), was dispensed onto the gasket well on the slides and were placed in a hybridization oven with rotation at 10 r.p.m. at 65°C for 17 h. After washing steps of the slides, scanning was performed with an Axon Scanner 4000B.

*Microarray validation by qRT-PCR.* The same samples used for the microarray analysis were analyzed by qRT-PCR (Pajuelo *et al*., 2014) to calculate the expression of 12 selected genes (primers listed in Supporting Information Table S3). The *recA* gene was used as standard and the fold induction ($2^{-\Delta\Delta Ct}$) for each gene was calculated according to Livak and Schmittgen (2001). The tested genes were classified as induced (fold change $\geq 2$), repressed (fold change $\leq -2$) and equally expressed ($2 >$ fold change $> -2$) genes comparing their expression in Δ*fur* versus wild-type or in iron-restriction versus iron-excess.

### In silico *analysis of putative Fur boxes*

The sequences of already described *V. vulnificus* Fur boxes (Ahmad *et al*., 2009) were used to scan the CECT4999 genome for potential Fur boxes with a maximum 30% of mismatches by using Ugene (Okonechnikov *et al*., 2012). We used subsystem annotation tools implemented in the SEED genomic platform (http://theseed.uchicago.edu/FIG/index.cgi) (Overbeek *et al*., 2005) to search for genes implicated in virulence and survival and manually extract the putative Fur boxes. WebLogo (Crooks *et al*., 2004) was used to build a consensus sequence logo, in which the height of individual letters within a stack of letters represents the relative frequency of that letter at a given position, and the overall height of the stack represents the degree of conservation at that position.

### In vivo *assays*

*Animal maintenance*. Three populations of farmed European eel (*Anguilla anguill*a) of 10 g, 20 g 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°C for a week. Six to 8-week-old BALB/c mice were purchased from Harlan Laboratory Models S.L. and maintained for 48 h in 100 l plastic cages with water and feed supplied by the Animal housing facilities of the University of Valencia (UV).

*Virulence.* Virulence for eels (10 g of body weight) was determined after immersion (normal or iron-overloaded eels [preinjected with 9 μg/g of $FeCl_3$) or i.p. injection according to Amaro *et al*. (1995). Virulence for mice was tested by i.p.

injection of normal and iron-overloaded (pre-injected with $FeCl_3$ [9 μg/g of mouse] 2 h before challenge) mice (BALBc, 20 g of body weight) according to Amaro *et al*. (1994). For both eels and mice, a total of six animals were used per control, strain and dose and were maintained in independent cages or tanks. Animal mortality was recorded for one week and was only considered if the inoculated bacterium was re-isolated in pure-culture from the moribund animal. Virulence (Lethal Dose 50% or $LD_{50}$) was calculated according to Reed and Muench (1938) and was expressed as CFU/g (i.p. injection) or ml of infective bath (immersion challenge).

*Colonization.* A total of 24 eels per strain were infected by immersion with the $LD_{50}$ of the wild-type strain and additional six eels were immersed in a control bath (PBS-1) for 1 h. Then eels were captured and placed in independent tanks per strain for 1 week. A total of 12 live eels were randomly sampled at 0, 9, 24, and 72 h post-infection, at a ratio of 3 per sampling point (Pajuelo *et al*., 2014). The rest of the eels were monitored for 1 week to check that the mortality was around 50%. Samples for determination of bacterial numbers on TSA-1 were taken from blood, head kidney, liver, spleen and gills, according to (Pajuelo *et al*., 2014) and bacterial counts were expressed as CFU/ml (blood) or CFU/g.

All the protocols were reviewed and approved by the Animal Ethics Committee of the University of Valencia.

## In vitro *assays*

*Induction of the VBNC state and resuscitation.* Cells from overnight cultures in MSWYE were transferred to ASW, ASW + Fe or ASW − $Fe_{(D)}$ (28°C, 160 r.p.m.) and were incubated at 4°C with shaking (60 r.p.m.) until no bacteria were detectable by drop plating on CM9A (induction of VBNC state). Then, cultures were incubated at 22°C with shaking (160 r.p.m.) and the resuscitation was monitored again by drop plating on CM9A. Selected genes from the microarray results were quantified by qRT-PCR during the induction of the VBNC state. The genes and the primers are indicated in Supporting Information Table S5.

*Motility and chemotaxis toward blood components.* Motility was assayed on Motility agar (CM9 agar [CM9A] 0.3% agar wt/vol) plus 100 μM $FeCl_3$ (MA + Fe) or plus 20 μM of D (MA − $Fe_{(D)}$) by inoculating 5 μl from an exponential phase culture (6 h). Plates were incubated at 28°C for 24 h and the surface of the bacterial mass on the plate ("colony") in $cm^2$ (SC) as well as the number of bacteria forming the "colony" in CFU (NB) were determined. Then, the parameter *motility rate* (Mr) was calculated as SC/log NB. In parallel, microscopic observations of bacterial suspensions were made in a Nikon Phase-Contrast Microscope.

Human-, eel- and hemolytic-eel-plasma (HP, EP and HEP) were obtained as described previously (Pajuelo *et al*., 2014). The chemotactic response (Cr) toward plasma was determined by using the capillary assay as described by Larsen *et al*. (2001). To this end, bacteria were recovered from cultures grown 6 h in CM9, washed twice in PBS and diluted in chemotaxis buffer (PBS, 0.01 mM EDTA) at $10^7$ CFU per ml (Larsen *et al*., 2001). Then, volumes of 0.5 ml of bacterial suspension were dispensed in 1.5 ml tubes that were put into contact with HP, EP, HEP or chemotaxis buffer (control) contained in capillary tubes (5-μl pre-calibrated pipettes; Vitrex) for 35 min. The number of bacteria inside the capillaries was determined on CM9A plates and the chemotaxis toward HP, EP or HEP was expressed as the ratio between bacterial numbers in the corresponding capillaries versus control capillaries.

*Biofilm formation.* Bacteria were grown in 96-well plates (NUNC) containing 200 μl of LB-1, supplemented or not with either $FeCl_3$ 100 μM or 20 μM of D and biofilm production was quantified by staining with crystal violet (Jones *et al*., 2008) at specific time intervals.

*Growth in iron-restriction and in presence of hemin and Tf as the sole iron-sources.* Growth in CM9-Hm and CM9 − $Fe_{(Tf)}$ was monitored as previously described (Pajuelo *et al*., 2014).

*Minimal inhibitory concentration (MIC) of iron chelators, microcide peptides, and saponin.* MICs for polymyxin B sulfate (Sigma), lysozyme (Sigma) and sodium deoxycholate (SDC) (Sigma) were determined in CM9A, CM9A + Fe and CM9A − $Fe_{(D)}$ plates. Plates were inoculated with exponential phase cultures (6 h) in CM9, CM9 + Fe or CM9 − $Fe_{(D)}$ and sterile disks impregnated with different concentrations of polymyxin B sulfate (1–$10^3$ μg/ml), lysozyme (1–$10^3$ μg/ml), or SDC ($10^3$–$10^6$ μg/ml) were added. The MIC was defined as the lowest substrate concentration at which there was not growth. In the case of saponin (Sigma), 96-well plates (NUNC) containing 200 μl of PBS + saponin (100 μg/ml), were inoculated with overnight cultures in CM9 in a ratio 1:100 (v/v), were incubated at 28°C with shaking (160 r.p.m.) and viable bacterial count on CM9A was performed at 5, 10, 15, 20, and 30 min post-incubation by the drop-plate method (Hoben and Somasegaran, 1982).

*Resistance to acid, oxidative, and nitrosative stresses.* Washed bacteria from overnight cultures in CM9 or CM9 − $Fe_{(D)}$ at 28°C were inoculated in tubes containing 5 ml of MSWYE (control), MSWYE-pH5 (acid-stress) or PBS-$H_2O_2$ (0.1% vol/vol) (oxidative stress), supplemented or not with 20 μM Dipyridyl, at a ratio of $10^5$ CFU/ml. Tubes were incubated at 28°C with shaking (60 r.p.m.) for 180 min. Viable bacterial counts were estimated by drop plating on CM9A at intervals of 30 min. To test resistance to nitrosative stress, bacteria from overnight cultures in CM9, CM9 + Fe or CM9 − $Fe_{(Tf)}$ at 28°C were washed in PBS and inoculated in the respective fresh medium supplemented or not with 400 μM DPTA NONOate (NO donor, Cayman Chemical, Michigan, USA). DPTA NONOate was decay for 3 h in the medium in order to achieve a constant NO release (Henares *et al*., 2012). Growth was followed by measuring absorbance at 600 nm on a spectrophotometer (Thermo scientific) at 1-h intervals for 8 h.

*Proteolytic and hemolytic activity.* The extracellular products (ECP) from the three strains were obtained as previously described (Biosca and Amaro, 1996) from overnight cultures on CM9A − Fe or CM9A − $Fe_{(D)}$ and the maximal dilution of ECP with positive activity on agarose-erythrocytes (bovine erythrocytes from Sigma) or agarose-casein was determined. In parallel, the hemolytic activity of live cells at short term was determined according to Shinoda *et al*. (1985).

*Cell-associated polysaccharides.* Crude fractions of cell-associated polysaccharides (LPS plus capsule) were obtained from overnight cultures of the three strains in CM9 + Fe or CM9 − $Fe_{(Tf)}$ as described by Hitchcock and Brown (1983). The polysaccharide concentration was determined with Total Carbohydrate Assay Kit (BioVision) as described by the manufacturers and samples were adjusted to 0.2 μg/μl of polysaccharide. 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 (Bio-Rad, Madrid, Spain) (Towbin *et al.*, 1979) and subjected to immunoblot analysis. The membranes were probed with serovar E-specific sera (Amaro *et al.*, 1992) diluted 1:3000 and were developed following incubation with anti-rabbit IgG HRP-conjugated secondary antibody diluted 1:10 000 (Sigma), using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Madrid, Spain).

*Statistical analysis.* All the experiments, except the virulence assays, were performed by triplicate. Statistical analysis was performed using the SPSS 17.0 for windows. The results are presented as means ± SE (standard error of the means). The significance of the differences between means was tested by using the unpaired Student's t-test with a $P < 0.05$. When the effects of more than two independent variables were taken into account, a GLM (general linear modeling) univariate analysis was performed. This analysis allows to highlight not only the significance of the single variables but also that of their interactions.

A. Effect of strain (wild-type vs mutant), iron (with vs without) and pH (neutral vs acid) on bacterial growth at 2, 4 and 6 h post-incubation
B. Effect of strain (wild-type vs mutant), iron (with vs without) and NO (with vs without) on bacterial growth at 1 4 and 7 h post-incubation

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

**Fig. S1.** Categories of gene regulation after by comparing iron-stimulon with Fur-regulon. **(A)** Number of genes differentially expressed (DEG) by *V. vulnificus* per regulation category and replicon (two chromosomes and one plasmid). The categories are represented in a colors code. **(B)** Most significant motif derived from identified putative Fur boxes among virulence and survival-related genes studied returned by the MEME tool (Bailey *et al.*, 2009). The height of each letter represents the relative frequency of each base at different position in the consensus sequence.
**Fig. S2.** Hemolytic activity of *V. vulnificus* strains at short term. Absorbance of the supernatant of a bovine erythrocyte's suspension in PBS (1% vol/vol) incubated with bacterial cells **(A)** or bacterial extracellular products (ECP) **(B)** was measured at 520 nm.
**Table S1**. List of genes included in the iron-stimulon in *V. vulnificus* CECT4999. Only values of fold change $\geq$2/$\leq$-2 were considered, with a p-value cut-off of 0.01 (+, gene up-regulated in iron-rescrition; -, gene down-regulated in iron restriction).
**Table S2**. List of genes included in the Fur-regulon in *V. vulnificus* CECT4999. Only values of fold change $\geq$2/$\leq$-2 were considered, with a p-value cut-off of 0.01 (+, gene up-regulated in Δ*fur*; -, gene down-regulated in Δ*fur*).
**Table S3.** Comparison of fold change values obtained by hybridization with the *V. vulnificus* array and by qRT-PCR.
**Table S4.** Results of the GLM (general linear modeling) univariate analysis of variance
**Table S5.** Identified putative Fur boxes among virulence and survival-related genes studied.