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Heme, an iron supply for vibrios pathogenic for fish

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Abstract One of the main mechanisms present in gram-negative bacterial pathogens to obtain iron is the utilization of free heme or heme proteins from the host tissues. *Vibrio anguillarum*, the etiological agent of vibriosis in fish, and *Photobacterium damselae* subsp. *piscicida*, the causative agent of fish pasteurellosis, can acquire iron from free heme or heme-containing proteins present in the host tissues by a siderophore-independent mechanism. Similarly to other animal and human pathogens, the general mechanism for heme uptake in these two species consists in the presence of an outer membrane receptor that transport the heme molecule into the periplasm via a TonB-dependent process, and additional proteins that complete the transport of heme from the periplasm into the cell cytoplasm. Expression of heme uptake genes is iron-regulated at the transcriptional level by the repressor protein Fur. The heme uptake mechanisms are believed to contribute to virulence for fish. The existence of variability in the distribution of heme transport genes among strains suggests that gene inactivation and/or horizontal transfer might play a significant role in generating intraespecific genetic diversity.

Keywords *Vibrio anguillarum* · *Photobacterium damselae* · Fish pathogens · Iron uptake · Heme uptake · Virulence

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Introduction

Owing to their abundance in the host, heme and heme-containing proteins are potential source of iron for invading microorganisms. Thus, one of the main mechanisms present in gram-negative bacterial pathogens to obtain iron is the utilization of heme or heme-containing proteins from the host tissues (Wandersman and Delepelaire 2004). In this regard, fish species are not very different from other vertebrates, and it is clear now that bacterial fish pathogens follow similar strategies to other animal and human pathogens to invade the host and cause disease. Our work has been focused in the last years in the study of the iron uptake systems in bacterial fish pathogens belonging to the *Vibrionaceae* family, with special emphasis to *Vibrio anguillarum* and *Photobacterium damselae*. The current knowledge of the heme uptake systems present in these two species is summarized in this review.

The mechanism of heme iron utilization in these fish pathogens is believed to follow the

general model of heme uptake in gram-negative bacteria. Free heme is not encountered in vivo, and thus it is expected that bacteria acquire heme from hemoproteins. Hemoproteins are recognized by iron regulated outer membrane protein receptors, and the heme moiety is unloaded and transported across the outer membrane. The heme receptors must be able to extract heme from different carrier proteins prior to the transport step. How the heme receptor interacts with the heme-protein complex to release the heme ligand is a process not yet fully understood. Some insights have been gained in experiments with *Yersinia* and *Neisseria* heme receptors, and amino acid residues in the receptor that are crucial for its interaction with the heme-protein complex have been identified (Bracken et al. 1999; Perkins-Balding et al. 2003). Transport of heme across the outer membrane is an active process that uses energy of the cytoplasmic membrane to bring heme into the periplasm, and this energy is provided by a Ton system (Stojiljkovic and Hantke1992; Henderson and Payne1994a). The TonB system consists of the cytoplasmic membrane proteins ExbB and ExbD, and the cytoplasmic membrane anchored protein TonB, that together are responsible for the transduction of the cytoplasmic membrane proton-motive force to the receptors. A conserved region in the outer membrane receptors known as *TonB box*, is responsible for the interaction with TonB. Interaction of TonB with outer-membrane receptors is believed to induce a conformational change of the receptors, resulting in the transport of bound ligands into the periplasmic space (Skare et al. 1993). Once in the periplasmic space, heme is bound by a periplasmic heme-binding protein. Then, a permease and an ATP-hydrolysing protein, conform an inner-membrane associated ABC-transporter which finally transports heme into the cytoplasm through an ATP dependent process (Fath and Kolter 1993). The fate of heme after entering the bacterial cytoplasm is not fully understood. Although some gram-negative bacteria use heme oxygenase-like enzymes to acquire iron from heme (Zhu et al. 2000; Ratliff et al. 2001), no heme oxygenases have been described to date in *Vibrionaceae* species.

Heme uptake in *V. anguillarum*

Vibrio anguillarum is the etiological agent of vibriosis, a septicemic disease that affects a large number of marine fish species, as well as bivalve molluscs and crustaceans (Toranzo and Barja 1990). Sørensen and Larsen grouped *V. anguillarum* isolates in 10 serogroups based on antigen ''O'' (Sørensen and Larsen 1986). The number of O-serotypes has been later extended up to 23 (Pedersen et al. 1999). However, only serotypes O1 and O2 and, to a lesser extent, serotype O3 are considered important pathogens since most vibriosis outbreaks described so far were caused by one of these serotypes. (Toranzo and Barja 1990; Larsen et al. 1994). Pathogenic strains of *V. anguillarum* can acquire iron from heme and heme-containing proteins, including hemoglobin and hemoglobin–haptoglobin, by a siderophore-independent mechanism (Mazoy and Lemos 1991). Acquisition of iron from heme is believed to be facilitated by the production of hemolysins or cytotoxins that can lyse host cells and release intracellular heme (García et al. 1997), and several hemolysin genes have been described in *V. anguillarum* strains to date (Hirono et al. 1996; Rodkhum et al. 2005; Rock and Nelson 2006).

Vibrio anguillarum cells bind hemin and hemoglobin

The existence of hemoglobin- and hemin-binding activities in *V. anguillarum* was first demonstrated in strains of serotypes O1 and O2, by performing dot-blot assays using biotinylated conjugates (Mazoy and Lemos 1996). Recently, we have also shown that the heme-binding ability is extended to all *V. anguillarum* strains tested, independently of the O-serotype, origin or phenotypic characteristics (Mouriño et al. 2005). Binding showed to be independent of the iron load in the culture medium. In addition, pre-treatment of cells with proteinase K reduced drastically the heme-binding ability (Fig. 1). These data indicated that heme binding activity in this species could be exerted by constitutive outer membrane proteins. Interestingly, hemoglobin-binding was inhibited by previous incubation of cells with hemin, indicating that the heme moiety is recognized by

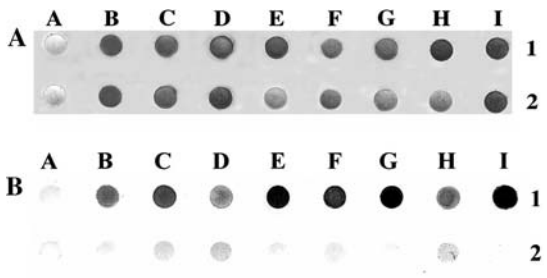

Fig. 1 (**A**) Hemin binding by whole cells from different strains of *V. anguillarum* cultured under iron-rich (1) and iron deficient (2) conditions. (**B**) Effect of proteinase K treatment (2) on hemin binding compared with non-reacted cells (1). A: *E. coli* HB101 (negative control); *V. anguillarum* strains (see Table 2 for serotype and gene content): B: 775; C: RV22; D: ET-208; E: RPM 41.11; F: ATCC 43309; G: ATCC 43310; H: ATCC 43311; I: ATCC 43313. Extracted from Mouriño et al. (2005)

the heme-binding molecules. Moreover, the fact that no hemoglobin degradation activities could be found in cell-free supernatants of *V. anguillarum*, supports the hypothesis that a direct interaction of the cell surface receptor with hemoglobin must be necessary for utilization of heme as iron source (Mazoy and Lemos 1991). An outer membrane protein of 39 kDa in serotype O1 strains, and of 37 kDa in serotype O2 strains were isolated as hemin and hemoglobin binding proteins, using an affinity purification methodology, although several other membrane proteins are likely involved in heme binding (Mazoy and Lemos 1996; Mazoy et al. 1996).

The heme uptake system

A *V. anguillarum* cosmid library was used to select genes that enabled *E. coli* 101ESD (*ent*) to utilize hemin as a sole iron source. As a result, the *huvA* gene encoding a protein with high homology to the heme receptors of *V. cholerae* and *V. vulnificus* was characterized, and it was shown that point mutations in this gene abolished heme and hemoglobin utilization as the sole iron source in *V. anguillarum* (Mazoy et al. 2003). The putative heme receptor protein, HuvA, has a predicted molecular weight of 79 kDa, whereas the hemin and hemoglobin binding proteins isolated from this species are 39 and 37 kDa proteins (see above). In addition, the *huvA* mutant still showed hemin and hemoglobin binding activity at levels compared to the wild type (Mazoy et al. 2003). These results altogether indicate that the heme receptor is not the only protein involved in hemin binding, and support the hypothesis that additional proteins can bind hemin although they likely do not function as heme transporters (Mazoy and Lemos 1996; Mazoy et al. 1996).

The gene coding for the heme receptor HuvA was sufficient to allow the use of heme as porphyrin source in an *E. coli hemA* strain (Mouriño et al. 2004). Thus, as described for other outer membrane heme receptors, the *V. anguillarum* HuvA protein is able to transport the entire heme molecule into the cytoplasm. HuvA contains conserved features that are common to other TonB-dependent outer membrane heme receptors. These include a TonB box similar to *V. vulnificus* HupA and *V. cholerae* HutA TonB boxes, as well as a conserved FRAP box, a motif that is conserved among outer membrane heme receptors. HuvA also contains a conserved phenylalanine residue at the C-terminal region.

The *huvA* gene is linked to eight additional genes in the chromosome of *V. anguillarum*, comprising a cluster that encodes the heme uptake and utilization system (Fig. 2). This system is constituted by nine heme transport and utilization proteins: HuvA, the outer membrane heme receptor; HuvZ and HuvX; TonB1, ExbB1 and ExbD1; HuvB, a periplasmic binding protein; HuvC, the inner membrane permease; and HuvD, the ABC-transporter ATP-ase (Mouriño et al. 2004).

To determine the role of these genes in heme utilization by *V. anguillarum* serotype O1, we constructed an in-frame deletion of the nine-gene cluster. When these genes were eliminated, *V. anguillarum* was impaired for growth with heme or hemoglobin as the sole iron sources (Mouriño et al. 2004). Construction of single gene in-frame deletions demonstrated that each of *huvAZBCD* genes are essential for heme utilization as iron source (Fig. 3). HuvB, in one side, and HuvCD in the other side, are homologous to described periplasmic heme transporters and inner membrane heme ABC-transporters, respectively (Occhino et al. 1998). Interestingly, deletion

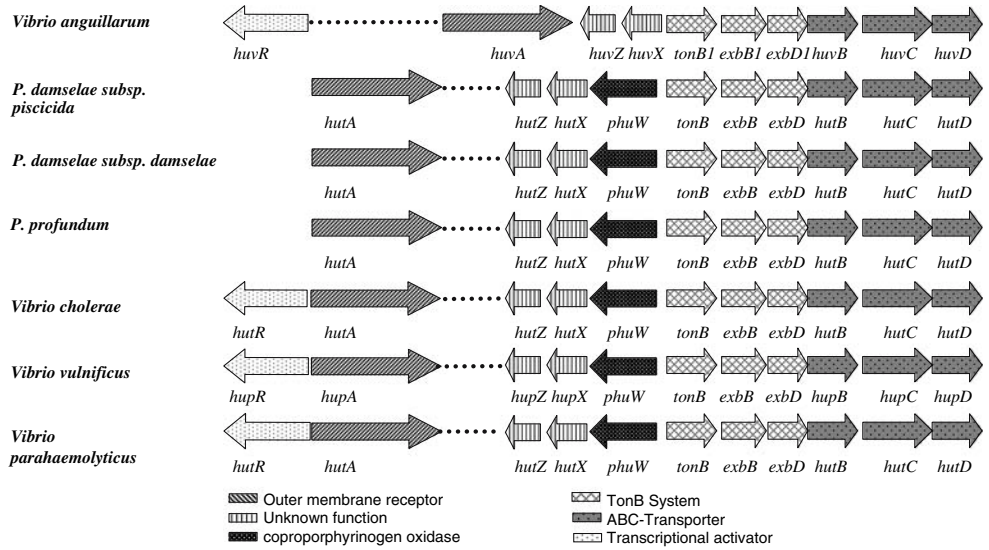

Fig. 2 Comparative chromosomal arrangement of heme uptake cluster genes in *Vibrionaceae*

of *huvBCD* homologues in *V. cholerae* does not completely abolish the use of heme iron, suggesting that the roles of these proteins can be partially fullfilled by other transporters.

Mutation of the *V. anguillarum huvZ* gene leads to a severe impairement for growth with heme as sole iron source. The role of HuvZ is not known, and homologues of this protein do not have a well known function either. It was reported that the *V. cholerae* HutZ protein binds heme with high efficiency and might act as a heme storage protein (Wyckoff et al. 2004). Less is known about the role of HuvX and its homologues. Deletion of *huvX* did not cause a significant decrease in the ability of *V. anguillarum* to utilize hemin and hemoglobin as the unique sources of iron (Fig. 3). Thus, we speculated that it could have a regulatory role on transcription of the heme uptake cluster genes. However, the transcriptional activity of the *huvA*, *tonB* and *huvX* promoters did not show significant differences between a parental strain and a *huvX* mutant (Mouriño et al. 2006).

Reverse-Transcriptase PCR assays as well as primer extension analysis, demonstrated that the *V. anguillarum* heme uptake cluster is arranged into three transcriptional units: *huvA*, *huvXZ*, and *tonB1exbB1D1-huvBCD* (Mouriño et al. 2006). Regulation of most iron transport systems in bacterial species occurs through the action of the repressor protein Fur. This protein requires the ferrous iron as a cofactor for dimerization and DNA binding (Hantke 1981). The analysis of the β-galactosidase activities of transcriptional fusions of the *V. anguillarum huvA*, *huvX* and *tonB1* promoters to a promoterless *lacZ* gene in both *V. anguillarum* wild-type and *fur* mutant strains demonstrated that iron is a major regulator of the gene expression of the heme uptake genes, and that this regulation is exerted by the Fur repressor (Mouriño et al. 2006) (Fig. 4). In congruence with the results of the transcriptional fussions, putative Fur boxes upstream of the *V. anguillarum huvA*, *huvX* and *tonB1* genes were identified (Mouriño et al. 2006).

The *V. anguillarum* heme uptake gene cluster was subcloned and different gene combinations were transformed into the *E. coli* strain 101ESD (*ent*), which is unable to synthesize siderophores and in addition lacks a genetic system for heme uptake. We found that the utilization of heme as an iron source in *E. coli* 101ESD requires *tonBexbBDhuvBCD* genes in addition to *huvA*, whereas the *huvXZ* genes are expendable (Mouriño et al. 2004). Since *huvZ* was essential for heme iron utilization in *V. anguillarum*, these results suggest that *E. coli* 101ESD might encode a function, which plays the role of HuvZ.

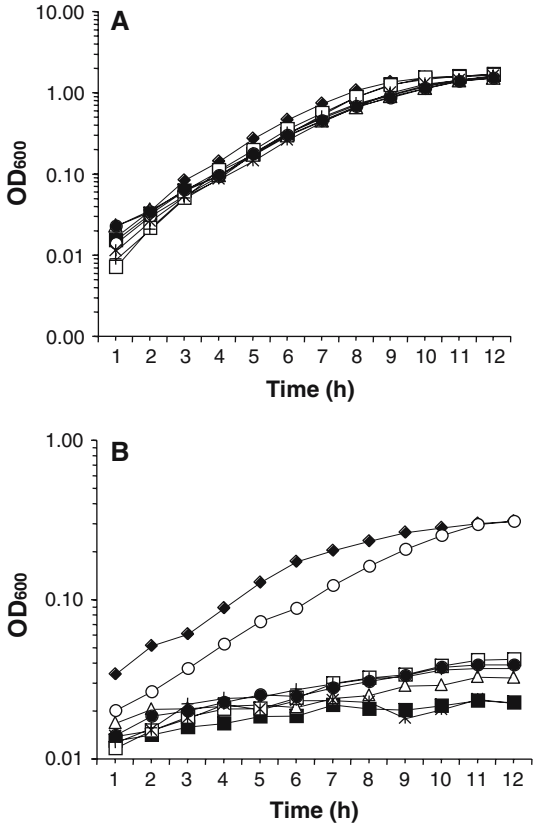

Fig. 3 Growth of *V. anguillarum* H-775-3 (♦), and the derivative mutants Δ*huvA* (■), Δ*huvZ* (Δ), Δ*huvX* (○), Δ*huvB* (□), Δ*huvC* (•), Δ*huvD* (+) and Δ*huv* (*) in CM9 minimal medium. Growth with hemin (10 μM) as iron source without (A) and with (B) free iron chelator 2,2′-dipyridyl (100 μM). Results are expressed as the average of three independent experiments. OD: Optical density. Extracted from Mouriño et al. (2004)

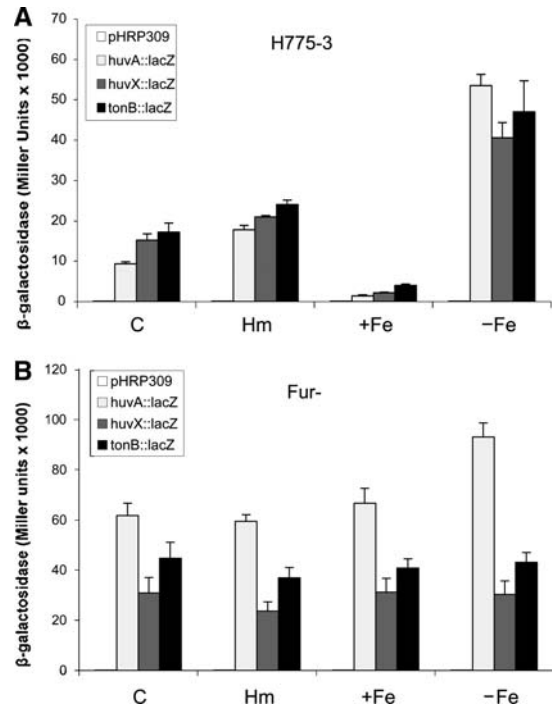

Fig. 4 Transcriptional analysis of the *huvA*, *huvXZ* and *tonB1exbB1D1-huvBCD* promoters with *lacZ* fusions assayed in *V. anguillarum* parental strain H775-3 (**A**) and in 775met11 *fur* mutant (**B**). Bars indicate *β*-galactosidase activities of cultures grown under different conditions: C, control conditions in CM9 medium; Hm, CM9 iron-depleted by addition of EDDA 1.5 μM, plus hemin 10 μM; +Fe, CM9 iron-replete by addition of $Fe_2(SO_4)_3$ 40 μM; –Fe, CM9 iron-depleted by addition of EDDA 1.5 μM

Two sets of *tonB* genes function in heme transport in *V. anguillarum*

The *V. anguillarum tonB1exbB1exbD1* genes, which are part of the heme uptake cluster, are not essential for heme utilization as an iron source, since a *tonB1* mutant did not show any phenotypic difference in iron uptake with heme as iron source (Stork et al. 2004). This suggested that a second set of *tonBexbBexbD* genes might be present in the *V. anguillarum* genome, as reported in *Vibrio cholerae* (Occhino et al. 1998) and in other *Vibrio* species (O'Malley et al. 1999). Using Tn*10* mutagenesis, a mutant was identified that was unable to use heme as sole iron source. In this way, it was identified a set of *tonB2exbB2exbD2* genes in *V. anguillarum* 775. A *tonB1-tonB2* double mutant was constructed, and showed to be impaired in transport of heme. Additional experiments and bioassays using different iron sources, demonstrated that both *tonB1* and *tonB2* genes are involved in heme uptake, whereas only *tonB2* is essential for the transport of siderophore anguibactin (Table 1) and seems to be a key element for multiplication into the host (Stork et al. 2004).

Recent similar experiments with *V. anguillarum* plasmidless strains belonging to serotype O2 demostrated that two TonB systems are also present in these strains (S. Mouriño, C.R. Osorio and M.L. Lemos, unpublished data). Similarly to the results obtained with 775 strain, only *tonB2* is

Table 1 Involvement of TonB1 and TonB2 systems of *V. anguillarum* in iron transport

Strain	Iron source					
	FAC[a]	Ang[b]	Vac[c]	Ent[d]	Fer[e]	Heme
V. anguillarum 775 (O1)	+[e]	+	–	+	+	+
tonB1$^-$	+	+	–	+	+	+
tonB2$^-$	+	–	–	–	+	+
tonB1$^-$ *tonB2*$^-$	+	–	–	–	–	–
V. anguillarum RV22 (O2)	+	–	+	+	+	+
tonB1$^-$	+	–	+	+	+	+
tonB2$^-$	+	–	–	–	+	+
tonB1$^-$ *tonB2*$^-$	+	–	–	–	–	–

Utilization of different iron sources by *tonB1*, *tonB2* and *tonB1tonB2* double mutants from *V. anguillarum* 775 and RV22

[a] Ferric amonium citrate

[b] Anguibactin from *V. anguillarum* 775

[c] Vanchrobactin from *V. anguillarum* RV22

[d] Enterobactin from *E. coli* HB101

[e] Ferrichrome

involved in the transport of vanchrobactin, the specific siderophore of *V. anguillarum* strains lacking pJM1 plasmid (Soengas et al. 2006), whereas heme can be transported using either TonB1 or TonB2 systems (Table 1). When each one of these systems are mutated the virulence degree for fish decreases, but clearly the double mutant is totally avirulent. This indicates the key role that TonB systems play in *V. anguillarum* virulence.

Gene arrangement and genetic variability of *V. anguillarum* heme uptake system

The gene arrangement in the *V. anguillarum* heme transport locus of serotype O1 strains shows some differences with respect to homologous clusters described in other *Vibrio* species. In all the species of *Vibrionaceae* studied so far, the gene encoding the outer membrane heme receptor is not linked to the rest of heme transport genes. However, the *V. anguillarum huvA* gene lies immediately downstream of the *huvZ* gene, constituting the unique exception so far described (Fig. 2).

The *huvXZ* and *tonB1exbB1exbD1* genes were detected by hybridization and PCR in all the strains tested among a collection of *V. anguillarum* isolates from different O-serotypes. However, some strains failed to hybridize to the *huvA* and *huvBCD* probes, indicating that at least one other heme uptake system is present in *V. anguillarum* (Table 2) (Mouriño et al. 2005). It is noteworthy that the only genes universally present in all strains are the *tonB1exbB1D1* genes (Table 2). Analysis of the DNA sequence downstream of *huvZ* in strains that proved negative for the *huvA* heme receptor gene, led to the characterization of the *huvS* gene that codes for another heme receptor present in strains lacking *huvA* (Table 2) (Mouriño et al. 2005). Although *huvS* shares low nucleotide sequence identity with *huvA*, the flanking regions are nearly 100% identical between *huvS* and *huvA*-contaning strains, suggesting that one of these heme receptor genes could have been gained by horizontal transfer. The *huvS* gene cloned in a plasmid was sufficient to complement *V. anguillarum huvA* mutants for the use of heme and hemoglobin as the sole iron source, demonstrating that it is functionally exchangeable with *huvA*. In addition, *huvS* could substitute *huvA* in the *E. coli* 101ESD complementation assay (Mouriño et al. 2005). In contrast to what was observed in *V. cholerae* (Mey and Payne 2001), where multiple tonB-dependent heme receptors can be present in the same cell, the analysis of the presence of *huvA* and *huvS* in a collection of different strains demonstrated that both receptors do not coexist in the same strain of *V. anguillarum* (Table 2).

The role of the *V. anguillarum* heme uptake mechanism in the virulence for fish is still unclear. It was demonstrated that when fish are previously injected with hemin or hemoglobin, the presence of a heme uptake system constitutes an advantage for *V. anguillarum* to cause infection (Mazoy et al. 2003). Furthermore, as said above, the TonB systems involved in iron uptake from heme and siderophores seem to be essential to successfully invade the host and cause an infection.

Heme utilization in *P. damselae* subsp. *piscicida*

Photobacterium damselae includes strains classified into two distinct subspecies, subsp. *damselae*

Table 2 Occurrence of heme uptake genes (see text) in a collection of *V. anguillarum* strains from different serotypes

Strain (serotype)	Presence of[a]					
	huvA	*huvBCD*	*huvXZ*	TonB1 system	TonB2 system	*huvS*
775 (O1)	+	+	+	+	+	–
TM-14 (O1)	+	+	+	+	+	–
R-82 (O1)	+	+	+	+	+	–
96-F (O1)	+	+	+	+	+	–
ATCC[a] 14181 (O2α)	+	+	+	+	+	–
ATCC 43306 (O2α)	–	+	+	+	+	+
RV22 (O2β)	+	+	+	+	+	–
43-F (O2β)	+	+	+	+	+	–
PT-493 (O3A)	–	+	+	+	+	+
13A5 (O3A)	–	–	–	+	–	–
ATCC 43307 (O3A)	–	+	+	+	+	+
ATCC 11008 (O3A)	–	+	+	+	+	+
B1.1.2/4	–	–	–	+	–	–
ET-208 (O3B)	–	+	+	+	+	+
RPM 41.11 (O4)	+	+	+	+	+	–
ATCC 43308 (O4)	–	+	+	+	+	+
ATCC 43309 (O5)	+	+	+	+	+	–
ATCC 43310 (O6)	–	+	+	+	+	+
ATCC 43311 (O7)	–	+	+	+	+	+
ATCC 43312 (O8)	+	+	+	+	+	–
ATCC 43313 (O9)	+	+	+	+	+	–
ATCC 43314 (O10)	–	+	+	+	+	+

[a] Presence detected by PCR and confirmed by Southern Blot hybridizations

and subsp. *piscicida*. *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*) is the causative agent of fish pasteurellosis, a disease affecting wild and cultured marine fish worldwide (Magariños et al. 1996). *Photobacterium damselae* subsp. *damselae* (formerly *Vibrio damsela*), has been reported to cause wound infections and fatal disease in a variety of marine animals and humans (Buck et al. 1991; Morris et al. 1982). Both subspecies are 100% identical in their 16S rRNA, although they are easily differentiated by a number of phenotypic traits.

Photobacterium damselae subsp. *damselae*, is capable of utilizing hemoglobin as sole iron source in vitro (Fouz et al. 1994). Production of an extracellular toxin with hemolytic and cytolytic activities has also been described (Kreger et al. 1987; Fouz et al. 1993), which may contribute to acquire heme iron through release of host hemoproteins. Iron availability has likely a role in the virulence of this bacterium, since its inoculation in iron-overloaded animals significantly increases the virulence degree of virulent strains. Solid-phase dot-binding assays demonstrated the existence of constitutive cell surface-mediated binding activity for hemoglobin, regardless of the iron load in the medium (Fouz et al. 1994).

Heme binding by *P. damselae* subsp. *piscicida*

Photobacterium damselae subsp. piscicida is able to use hemin and hemoglobin as unique iron sources in vitro (Magariños et al. 1994). Whole cells of *P. damselae* subsp. *piscicida* are able to bind hemin, as well as protoporphyrin IX and biotinylated bovine hemoglobin (do Vale et al. 2002). Although the hemin binding activity is observed under iron-supplemented and iron-restricted conditions, iron limitation results in an increased binding of hemin in virulent strains (Fig. 5). However, the hemin-binding ability of avirulent strains showed not to be affected by the iron load of the medium. Proteinase K treatment of whole cells markedly reduced the binding of hemin, indicating that protein receptors located at the cell surface are involved in the binding. Dot-blot assays demonstrated that purified *P. damselae* subsp. *piscicida* total and outer membrane proteins were able to bind hemin regardless of the iron levels of the medium, with the outer

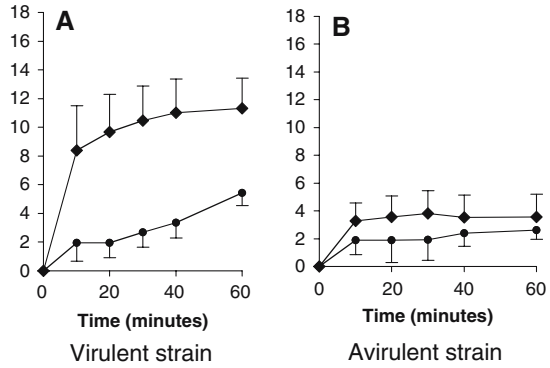

Fig. 5 Binding of Hemin by *P. damselae* subsp. *piscicida* virulent strain DI21 (**A**) and avirulent strain EPOY 8803-II (**B**). Cells grown in TSB + EDDHA (♦) or TSB + Fe (•) were tested, by a liquid binding assay. Extracted from do Vale et al. (2002)

membranes showing the strongest binding. Capsular polysaccharide also showed hemin binding activity, although with a much lower affinity than proteins. Heat treatment did not have significant effect in the binding ability of outer membrane proteins and capsular polysaccharides, suggesting that thermostable outer membrane proteins and capsular polysaccharides play a role in binding of hemin molecules in *P. damselae* subsp. *piscicida* (do Vale et al. 2002). Several studies report that lipopolysaccharides of other gram-negative bacteria also show hemin and/or hemoglobin binding activity (Bélanger et al. 1995; Grenier et al. 1997), although their role in further heme utilization is not fully understood.

We have thus demonstrated that in *P. damselae* subsp. *piscicida* and *V. anguillarum*, the hemin and hemoglobin binding activity via protein receptors is a constitutive factor, not regulated by iron, since binding properties are exerted at the same extent either in iron-restricted or in iron-enriched conditions. This is also the case for species as *V. vulnificus* (Fouz et al. 1996). By contrast, in *V. cholerae* and *V. parahaemolyticus*, the hemin- and hemoglobin-binding activities are iron-regulated (Henderson and Payne 1994b; Yamamoto et al. 1995).

Heme uptake genes

We have recently cloned and characterized the genetic determinants of heme utilization as iron source in strains of the two subespecies of *P. damselae* (Juiz-Río et al. 2005). This was acomplished by screening a gene library of *P. damselae* subsp. *damselae*, searching for genes that allowed *Escherichia coli* 101 ESD (*ent*) previously transformed with a plasmid containing the *V. anguillarum huvA* gene, to grow in the presence of hemin as the sole iron source. As a result, we identified a gene cluster which included the genes coding for proteins HutZ, HutX and HutW; TonB1, ExbB1 and ExbD1, the three components of the TonB system; HutB, the periplasmic binding protein; HutC, the inner membrane permease; and HutD, the ABC-transporter ATP-ase (Fig. 2). The aminoacidic sequence of all these proteins showed to be almost identical between both subspecies, and also very similar to homologous proteins described in *Vibrio* species. A gene coding for HutA, the outer membrane heme receptor, was also identified but it was not linked to the rest of the heme transport genes. We constructed an insertional mutant of *P. damselae* subsp. *piscicida*, inactivating the transcription of *hutCD* genes. The mutant strain showed a significantly reduced ability to grow in a medium with hemin and the iron chelator 2,2′-dipyridyl, thus demonstrating the role of this gene cluster in heme uptake.

The *hutA* gene encoding the outer membrane heme receptor, when transformed into *E. coli* EB53 *hemA* (a strain unable to synthesize porphyrins), was sufficient to confer this strain the ability to grow in the presence of hemin and hemoglobin as porphyrin sources (Fig. 6). The *P. damselae tonBexbBDhutBCD* genes in combination with *hutA* are necessary for heme utilization as an iron source in *E. coli* 101ESD (*ent*), whereas *hutZXW* are not (Fig. 6) (Juiz-Río et al. 2005). The actual role of *hutZXW* in heme iron utilization remains obscure. The *hutW* gene encodes a protein with homology to oxygen-independent coproporphyrinogen oxidases, an enzyme that converts coproporphyrinogen III into protoporphyrin IX, one of the steps in heme biosynthesis pathway (Panek and O'Brian 2002). However, its biological function has not specifically been demonstrated in any of the *hutW* homologues in *Vibrio* species. As reported in *Plesiomonas shigelloides*, *hutZXW* homologues

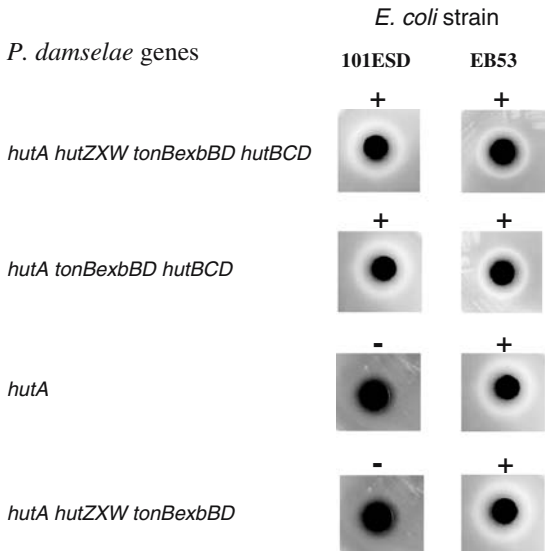

Fig. 6 Utilization of hemin as iron source by *E. coli* 101ESD and as porphyrin source by *E. coli* EB53 complemented with different combinations of *P. damselae* heme uptake genes

could play a role in preventing heme toxicity when *E. coli* is complemented with a heme uptake system and grown in the presence of heme (Henderson et al. 2001).

RT-PCR analyses showed that the heme uptake genes are arranged in three iron-regulated transcriptional units: *hutW-hutX-hutZ*, *tonB-exbB-exbD-hutB-hutC-hutD*, and *hutA*. Expression of RNA transcripts of the three transcriptional units in the heme uptake system of *P. damselae* was analyzed under normal, iron-rich and iron-limiting conditions using Reverse-Transcription-PCR, and it was demonstrated that *hutA*, *hutW* and *tonB* transcripts were weakly expressed under iron-rich conditions (Fig. 7). This observed iron-mediated regulation of the three promoters is in agreement with the existence of conserved putative Fur-binding sites upstream of the start codons of *hutA*, *hutW* and *tonB* genes (Osorio et al. 2004; Juiz-Río et al. 2005). The promoter region of *P. damselae hutA* contains a putative Fur-box (GATAATGATAGTAATTATC), with an identity of 16 out of 19 residues to the consensus Fur-box sequence described in *E. coli*. A Fur Titration assay verified that the Fur protein binds to the DNA region upstream of *hutA* (Juiz-Río et al. 2005). We recently identified the gene encoding the Fur protein in the two subspecies of *P. damselae*, and demonstrated that it acts as an iron-dependent transcriptional repressor (Juíz-Rio et al. 2004).

The genes encoding the heme uptake system of *P. damselae* are uniformly distributed in strains of the two subspecies (unpublished data). However, it is noteworthy that the *hutA* gene is intact in strains from subsp. *damselae* isolated from humans and a variety of fish species, but it is disrupted in the analyzed European and American strains of subsp. *piscicida*, constituting a pseudogene (Juiz-Río et al. 2005). In addition, the DNA sequence that flanks the *hutA* gene shows differences between strains and subespecies. Thus, the *hutA* gene of some subsp. *piscicida* strains is preceded by a ribosomal protein L25 gene, as it is in subsp. *damselae* strains, whereas a gene coding for a putative transposase is encountered downstream of the *hutA* stop codon, and it is absent from subsp. *damselae* strains (Fig. 8). The presence of this transposase could be related to insertion sequences or transposons, which would have played a role in the reorganization of its neighbouring DNA, thus leading to the generation of a *hutA* pseudogene.

A proposal for the existence of two distinct clonal lineages within *P. damselae* subsp. *piscicida*, represented by the European isolates in one hand, and the Japanese isolates in the other hand was reported recently (Magariños et al. 2000). The fact that the *hutA* pseudogene occurs in all the European and American isolates supports that idea of the two clonal lineages. In addition, since all the assayed subsp. *piscicida* strains can utilize heme as sole iron source (Magariños et al. 1994), the presence of a *hutA* pseudogene in some isolates suggests the existence of an additional, yet undescribed outer membrane heme receptor in those *P. damselae* subsp. *piscicida* strains.

Role of heme uptake in *P. damselae* virulence for fish

It was early demonstrated that heme utilization as an iron source could play a role in the virulence of *P. damselae* subsp. *piscicida* for fish. When hemin or hemoglobin were injected intra-

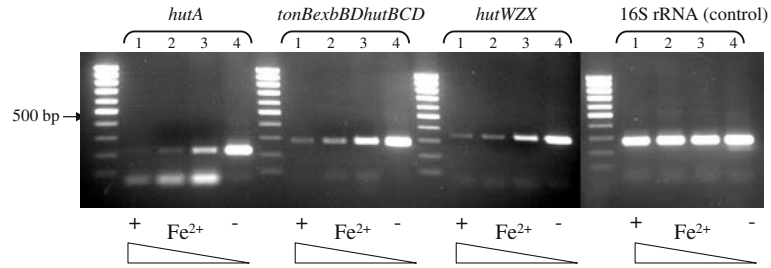

Fig. 7 Reverse-Transcription detection of mRNA in *P. damselae* ssp. *piscicida* MP7801 under different iron conditions. Lane 1: TSB + 10 μM $FeSO_4$; lane2: TSB; lane3: TSB + 100 μM 2,2′-dipyridyl; lane 4: TSB + 300 μM 2,2′-dipyridyl. The amplified fragments in each transcriptional unit are as follows: *hutA* transcript: a 204 bp fragment internal to *hutA* gene; *tonBexbBDhutBCD* transcript: a 260 bp fragment internal to *tonB* gene; *hutWZX* transcript: a 340 bp fragment internal to *hutX* gene; 16S rRNA transcript: a 267 bp fragment internal to 16S rRNA gene. Extracted from Juiz-Río et al. (2005)

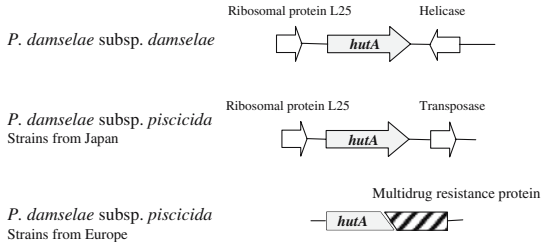

Fig. 8 Schematic representation of the genetic organization of *hutA* gene and neighboring DNA in three different type of *P. damselae* strains, depicting three distinct possibilities. Extracted from Juiz-Río et al. (2005)

peritoneally into fish a few hours before experimental infection, the lethality of the bacterium increased (Magariños et al. 1994). Using Reverse-Transcription PCR assay, we were able to detect mRNAs corresponding to the *tonBexbBexbDhutBCD* operon, in spleen and kidney specimens isolated from fish experimentally inoculated with *P. damselae* subsp. *piscicida* (S. Juiz-Rio, C.R. Osorio and M.L. Lemos, unpublished data). This suggests that the heme uptake system is expressed in vivo, and might significantly contribute to iron scavenging during the infective process.

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