# Binding of haemin by the fish pathogen *Photobacterium damselae* subsp. *piscicida*

**Ana do Vale[1,2,\*], Beatriz Magariños[1], Jesús L. Romalde[1], Manuel L. Lemos[3], Anthony E. Ellis[4], Alicia E. Toranzo[1]**

[1]Departamento de Microbiología y Parasitología, Facultad de Biología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain

[2]Institute for Molecular and Cell Biology, University of Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal

[3]Departamento de Microbiología y Parasitología, Facultad de Ciencias, Universidad de Santiago de Compostela, Campus de Lugo, 27002 Lugo, Spain

[4]Marine Laboratory, Fisheries Research Services, PO Box 101, Victoria Rd, Aberdeen AB11 9DB, Scotland, UK

ABSTRACT: Whole cells of virulent (DI 21 and B 51) and avirulent (ATCC 29690 and EPOY 8803-II) strains of *Photobacterium damselae* subsp. *piscicida*, grown under iron-supplemented or iron-restricted conditions, were able to bind haemin. Iron limitation resulted in an increased binding of haemin by DI 21, B 51 and ATCC 29690 cells but did not affect the haemin-binding ability of the EPOY 8803-II cells. Proteinase K treatment of whole cells markedly reduced the binding of haemin, indicating that protein receptors located at the cell surface are involved in the binding. This was confirmed by the observation that isolated total as well as outer membrane proteins from all the strains, regardless of the iron levels of the media, were able to bind haemin, with the outer membranes showing the strongest binding. Haemin binding by membrane protein extracts was not affected by heat treatment but was almost completely abolished by Proteinase K treatment, suggesting the presence of thermostable protein receptors for haemin. The capsular polysaccharide also appears to play a minor role in binding of haemin. It was concluded that constitutive as well as inducible mechanisms of haemin binding occur in *P. damselae* subsp. *piscicida*. These mechanisms would rely mainly upon the direct interaction between the haemin molecules and surface-exposed outer membrane protein receptors.

KEY WORDS: *Photobacterium damselae* subsp. *piscicida* · Haemin binding · Iron acquisition

## INTRODUCTION

Bacteria have a nutritional need for iron and must obtain it from the environment. One of the host defence mechanisms against infection is the ability to reduce free extracellular iron to concentrations below the minimum required to support the growth of bacteria (Martínez et al. 1990). Although there is plenty of iron present in the infected host, the amount of free iron, which is readily available to the infecting bacteria, is extremely small. This is due to the low solubility of $Fe^{3+}$ at physiological pH and to its sequestration by intracellular and extracellular high affinity iron-binding proteins (Bullen 1981, Griffiths 1987, Martínez et al. 1990, Payne 1993, Weinberg 1993, Schryvers & Stojiljkovic 1999).

Pathogenic bacteria have developed several mechanisms to acquire iron in order to overcome the iron-withholding capacity of the host. One involves the synthesis of siderophores and specific transport systems for the uptake of the iron-loaded siderophores into the cell (Otto et al. 1992, Moeck & Coulton 1998). Other mechanisms rely on the interaction between specific

*E-mail: avale@ibmc.up.pt

microbial receptors and host transferrin or haem-containing compounds (Otto et al. 1992, Lee 1995).

*Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*) is the causative agent of fish pasteurellosis, a disease known for a long time to affect wild and cultured marine fish in the USA and Japan (Kitao 1993). Since 1990, outbreaks of fish pasteurellosis have occurred in various European countries, causing important economic losses in gilthead seabream *Sparus aurata* and sea bass *Dicentrarchus labrax* L. cultures (Magariños et al. 1996b, Romalde & Magariños 1997).

It has been reported that iron plays a role in the virulence of *Photobacterium damselae* subsp. *piscicida* (Magariños et al. 1994). However, the mechanisms by which this fish pathogen can acquire iron from the host are not clearly understood. It has been shown that *P. damselae* subsp. *piscicida* is able to obtain iron bound to human transferrin by means of a siderophore-mediated mechanism (Magariños et al. 1994). Furthermore, it was shown that this bacterium is able to utilise haemin and haemoglobin as the sole iron source *in vitro* and that haemin and haemoglobin, as well as ferric ammonium citrate, injected intraperitoneally into the fish several hours before experimental infection increase the lethality of this microorganism (Magariños et al. 1994).

The aims of the present work were to investigate whether *Photobacterium damselae* subsp. *piscicida* is able to bind haemin and to elucidate the nature of the haemin-binding mechanisms.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Four strains of *Photobacterium damselae* subsp. *piscicida* (DI 21, B 51, ATCC 29690 and EPOY 8803-II) were used in this study. The origin and virulence of the strains are listed in Table 1. In some experiments, *Escherichia coli* HB

101 and *Vibrio anguillarum* 775 were used as controls. *P. damselae* subsp. *piscicida* and *V. anguillarum* strains were routinely cultured at 25°C in tryptic soy broth (TSB) (Pronadisa) or on tryptic soy agar (TSA) supplemented with NaCl to a final concentration of 1% (w/v) (TSB-1 and TSA-1, respectively). *E. coli* was cultured in TSB or on TSA at 37°C. All strains were stored frozen at –70°C in the appropriate broth media supplemented with 15% (v/v) glycerol.

Iron-limited conditions were obtained by adding the iron chelator ethylenediamine-di-(*o*-hydroxyphenyl-acetic acid) (EDDHA, Sigma) to the TSB-1 at a final concentration of 100 µg ml$^{-1}$. Iron-supplemented conditions were obtained by addition of FeCl$_3$ to the culture medium at a final concentration of 10 µM.

**Preparation of total and outer membrane proteins.** Bacterial strains were grown for 24 to 48 h in TSB-1 supplemented either with EDDHA or FeCl$_3$ and were then harvested by centrifugation at $10\,000 \times g$ for 10 min at 4°C. Total and outer membrane proteins were extracted as previously described (Toranzo et al. 1983). To obtain outer membrane proteins, total membranes were subjected to treatment with 3% (w/v) sodium lauroyl sarcosinate (sarcosyl, Sigma) to selectively solubilise inner membranes (cytoplasmic membranes). The outer membranes were then collected by centrifugation. Total and outer membrane proteins were stored at –20°C. The protein concentrations were determined using a Bio-Rad protein assay kit, with bovine serum albumin as standard.

**Haemin-binding assays.** Bovine haemin (Sigma) was dissolved in 10 mM NaOH to a final concentration of 1 mg ml$^{-1}$ before use. Haemin binding was assayed using 2 different procedures.

(1) Solid-phase dot blot assay. For the whole cell assay, bacteria were cultured for 24 to 48 h under iron-limited or iron-supplemented conditions and harvested by centrifugation at $4000 \times g$ for 5 min. They were then washed in Tris-buffered saline (TBS) (50 mM Tris-HCl supplemented with 0.9% NaCl, pH 8.0) and resuspended to an optical density (OD) of 1.0 at 580 nm. A 30 µl volume of the cell suspension was placed onto nitrocellulose membranes (0.45 µm pore size; Schleicher & Schuell) in a dot blot manifold. The binding of haemin was assayed as previously described (Mazoy & Lemos 1996). Briefly, after immobilisation the membranes were air dried, blocked with gelatin (2% in TBS), incubated for 2 h with haemin (10 µM in TBS), immersed for 30 min in 12.5% trichloroacetic acid, washed in distilled water and stained with 3,3′-dimeth-

Table 1. Origin and virulence of the *Photobacterium damselae* subsp. *piscicida* isolates used in the present study

| Strain | Origin | | Virulence |
| | Source | Donor[a] | for fish[b] |
| --- | --- | --- | --- |
| DI 21 | *Sparus aurata*, Spain | Laboratory collection[c] | + |
| B 51 | *Dicentrarchus labrax*, Spain | J. Borrego | + |
| EPOY 8803-II | *Epinephelus akaara*, Japan | K. Muroga | – |
| ATCC 29690 | *Seriola quiqueradiata*, Japan | ATCC | – |

[a]Donor addresses: ATCC (American Type Culture Collection), Rockville, Maryland; K. Muroga, Faculty of Applied Biological Science, Hiroshima University, Hiroshima, Japan; J. Borrego, Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Málaga, Spain
[b]Data obtained from Magariños et al. (1994)
[c]Departamento de Microbiología y Parasitología, Facultad de Biología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain

oxybenzidine (DMB, Sigma). The DMB solution (50 mg of DMB in 15 ml of distilled water) was freshly prepared just before staining, and after the solution was stirred for 15 min, 5 ml of a 0.5 M sodium citrate buffer (pH 4.4) and 100 µl of 30% $H_2O_2$ were added. After staining for 1 to 3 min, the membrane was washed with water to clear the background.

(2) Binding in liquid. The protocol used to detect binding of haemin in liquid was similar to that previously described (Hirst et al. 1994). Bacterial strains were cultured for 24 to 48 h under iron-supplemented and iron-limited conditions, collected by centrifugation at $4000 \times g$ for 5 min and washed in phosphate-buffered saline (PBS; pH 7.4). They were then resuspended in the same buffer and adjusted to an OD of 0.8 at 580 nm. Bovine haemin was added to the bacterial suspensions at a final concentration of 20 to 25 µg ml$^{-1}$ followed by incubation at 25°C for 60 min. At intervals, bacteria were pelleted in an Eppendorf centrifuge. Binding of haemin was determined by assaying spectrophotometrically at 400 nm the residual compound in the supernatant. The amount of residual haemin was then compared with a standard curve of haemin in PBS, and the amount of compound bound by the bacteria was then determined by subtraction. To determine whether haemin binding was protein associated, bacterial suspensions were incubated with 500 µg ml$^{-1}$ of Proteinase K (Sigma) in PBS for 1 h at 37°C before liquid-binding assays. Bacterial suspensions not preincubated with the enzymes were used as controls.

**Competitive binding assays.** Competitive binding assays were performed with bacteria cultured under iron-limited conditions to investigate whether different haem compounds shared the same binding site or receptor. Bovine haemoglobin and protoporphyrin IX (both from Sigma) were dissolved, to a final concentration of 1 mg ml$^{-1}$, in distilled water or 10 mM NaOH, respectively. Bacteria were incubated in the presence of 30 µg ml$^{-1}$ bovine haemoglobin or protoporphyrin IX for 1 h at 25°C. Bacteria were then pelleted by centrifugation and resuspended in PBS containing 30 µg ml$^{-1}$ haemin. The amount of haemin bound over a 60 min period was determined spectrophotometrically, as described above. Cells not pre-incubated with the competitors were used as controls.

**Haemin binding by bacterial membrane protein extracts.** To evaluate the binding of haemin by membrane protein extracts, volumes of 20 µl of either total or outer membrane proteins, containing 100, 10 and 1 µg ml$^{-1}$ of protein in distilled water, were immobilised on nitrocellulose membranes, which were then blocked with gelatine and incubated with haemin, immersed in 12.5% trichloroacetic acid, washed in distilled water and stained with DMB, as described above. *Vibrio anguillarum* 775 was used as a positive control

and *Escherichia coli* HB101 as a negative control. The effect of heat or protease treatment on haemin binding by protein extracts was evaluated by incubating bacterial total and outer membrane proteins, adjusted to a concentration of 100 µg ml$^{-1}$ in distilled water, either for 10 min at 100°C or with Proteinase K (added to a final concentration of 100 µg ml$^{-1}$) for 45 min at 37°C. The samples were then loaded onto a nitrocellulose membrane (2 µg per band) and subjected to a dot blot assay to evaluate the binding of haemin. Untreated samples were used as controls.

**Haemin binding by purified capsular polysaccharide.** Purified capsular polysaccharide from the pathogenic strain DI 21 was previously obtained (Bonet et al. 1994) and maintained, lyophilised, until used. This capsular material was resuspended in TBS to a final concentration of 1 mg ml$^{-1}$. The suspension was double diluted, until a concentration of 125 µg ml$^{-1}$ was obtained. From each concentration, 30 µl was immobilised on a nitrocellulose membrane and tested for the ability to bind haemin, using the dot blot assay. To investigate the effect of heat or protease treatment on the binding of haemin by purified capsular polysaccharide, the samples, resuspended in TBS at a concentration of 1 µg ml$^{-1}$, were incubated either at 100°C for 10 min or for 30 min at 37°C with Proteinase K (final concentration of 100 µg ml$^{-1}$). Treated suspensions were diluted as above and subjected to the dot blot assay to detect binding of haemin. Untreated samples were used as controls.

**Statistical analysis.** The results of the liquid binding assays were analysed by applying Student's *t*-test. The differences were considered significant when $p < 0.05$. The data are presented as mean ± standard error.

## RESULTS

### Haemin binding by whole cells

All the virulent and non-virulent strains tested, regardless of culture conditions, were able to bind haemin (Fig. 1a). However, it was found that when cells from the DI 21, B 51 (virulent) and ATCC 29690 (non-virulent) strains were grown under iron-limited conditions, the affinity for the compound increased greatly. Although this difference was observed using both methods, it was more evident when the assay in liquid was performed ($p < 0.005$). Fig. 1b compares the haemin-binding abilities of the virulent strain DI 21 under iron-restricted conditions with those under iron-supplemented conditions in the liquid assay. In contrast, growth under iron-limited conditions did not change the haemin-binding levels of the non-virulent EPOY 8803-II cells (Fig. 1c).
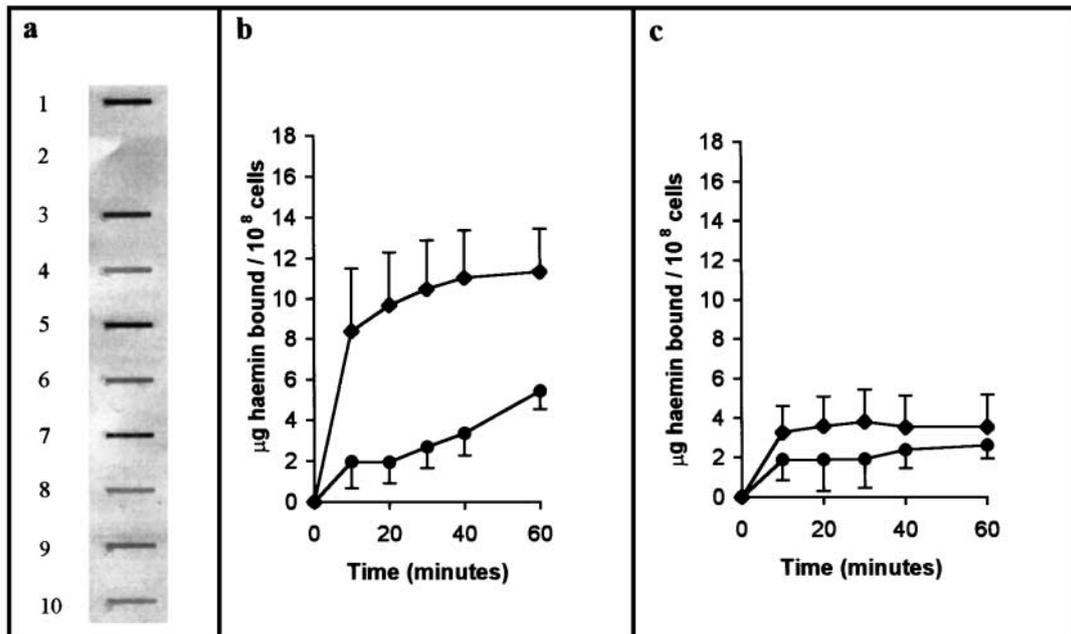
Fig. 1. Binding of haemin by *Photobacterium damselae* subsp. *piscicida* whole cells. (a) *P. damselae* strain DI 21(lanes 3, 4), B 51 (5, 6), ATCC 29690 (7, 8) and EPOY 8803-II (9,10) cells cultured under iron-restricted (tryptic soy broth with NaCL [TSB-1] + ethylenediamine-di-(*o*-hydroxyphenyl-acetic acid) [EDDHA]) (3, 5, 7, 9) or iron-supplemented (TSB-1+Fe) (4, 6, 8, 10) conditions were tested by the dot blot assay. *Vibrio anguillarum* 775 (1) and *Escherichia coli* HB101 (2) were used as positive and negative controls, respectively. Binding of haemin by *P. damselae* subsp. *piscicida* (b) strain DI 21 or (c) strain EPOY 8803-II. Cells grown under iron-restricted (◆) or iron-supplemented (●) conditions were tested by the liquid binding assay

## Haemin binding by bacterial membrane protein extracts

Using the dot blot assay, it was found that both total and outer membrane proteins from iron-supplemented or from iron-restricted *Photobacterium damselae* subsp. *piscicida* cells were able to bind haemin. With the same amount of protein, stronger binding to outer membrane proteins than to total proteins was detected (Fig. 2a).
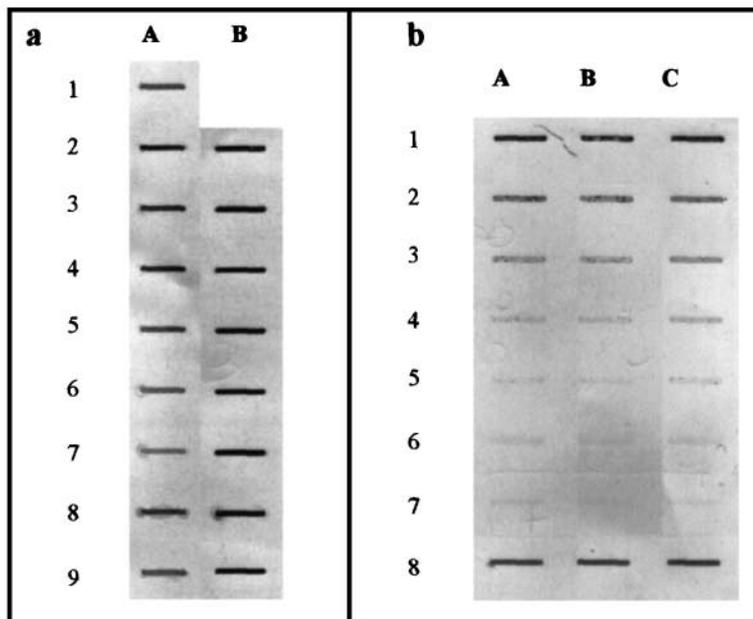


Fig. 2. Binding of haemin to *Photobacterium damselae* subsp. *piscicida* protein membrane extracts and purified capsular polysaccharide. (a) Binding of haemin to total (A) and outer (B) membrane protein extracts. Aliquots containing 2 μg of total (a, lane A) and outer (a, lane B) membrane proteins from strains DI 21 (2, 3), B 51 (4, 5), ATCC 29690 (6, 7) and EPOY 8803-II (8, 9) cultured under iron-restricted (2, 4, 6, 8) or iron-supplemented (3, 5, 7, 9) conditions were loaded onto the nitrocellulose membrane. *Vibrio anguillarum* 775 was used as a positive control (1). (b) Binding of haemin by DI 21 purified capsular polysaccharide. Lane A: untreated capsular polysaccharide. Lane B: heat-treated capsular polysaccharide. Lane C: capsular polysaccharide treated with Proteinase K. Rows 1 to 5: decreasing amounts of capsular polysaccharide (30 to 1.815 μg). Row 6: Proteinase K alone. Row 7: *Escherichia coli* HB101 (negative control). Row 8: *V. anguillarum* 775 (positive control). Haemin detected with 3,3′-dimethoxybenzidine

## Haemin binding by purified capsular polysaccharide

Because the presence of a constitutive capsular polysaccharide in the pathogenic *Photobacterium damselae* subsp. *piscicida* strains was previously reported (Bonet et al. 1994), we tested the ability of DI 21 capsular polysaccharide to bind haemin. When the dot blot assay was used, purified capsular polysaccharide was observed to bind haemin. Binding was detected when 30, 15, 7.5 or 3.75 µg of capsular polysaccharide was loaded on the membrane but it was no longer detectable when 1.8 µg or less per band was tested (Fig. 2b).

## Inhibition assays

When iron-limited or iron-supplemented bacterial cells were pre-treated with Proteinase K, their ability to bind haemin was greatly reduced compared with that of cells not treated with the enzyme (p ≤ 0.05, except for the samples collected after 10 min for which the difference was not significant) (Fig. 3a). The inhibitory effect of Proteinase K treatment was also ob-
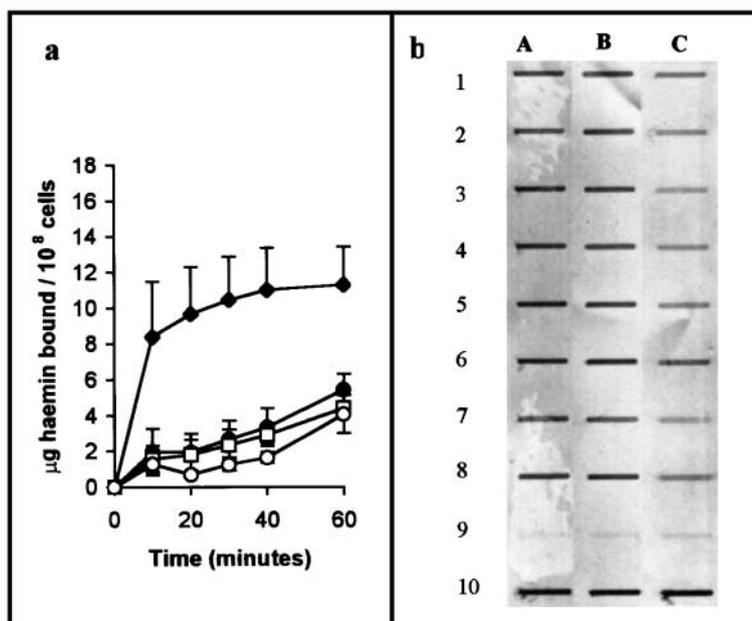


Fig. 3. (a) Binding of haemin by *Photobacterium damselae* subsp. *piscicida* DI 21. Cells grown under iron-restricted (□) or iron-supplemented (○) conditions were incubated with Proteinase K and subsequently tested for the binding of haemin. Untreated bacteria grown under iron-restricted (♦) or iron-supplemented (●) conditions were used as controls. (b) Binding of haemin to total protein extracts of *P. damselae* subsp. *piscicida* (lane A). Effect of heat treatment (lane B) and Proteinase K (lane C) on the binding activity. Aliquots containing 2 µg of protein from strains DI 21, B 51, ATCC 29690 and EPOY 8803-II cultured under iron-restricted (rows 1, 3, 5 and 7) or iron-supplemented (rows 2, 4, 6 and 8) conditions were used. Haemin was detected with 3,3′-dimethoxybenzidine. Row 9: *Escherichia coli* HB101 (negative control). Row 10: *Vibrio anguillarum* 775 (positive control)

served when total membrane proteins were tested for haemin binding in a dot blot assay (Fig. 3b). In contrast, binding of haemin to DI 21 capsular polysaccharide was found to be unaffected by pre-treatment with Proteinase K (Fig. 2b) Heating at 100°C for 10 min did not affect the ability of either total membrane protein extracts (Fig. 3b) or capsular polysaccharide (see Fig. 2b) to bind haemin.

Attempts to analyse the haemin binding by outer membrane proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blotting were unsuccessful (data not shown), perhaps indicating that the reducing conditions used destroyed the ability of the proteins to bind haemin.

## Competitive binding assays

Preliminary assays showed that *Photobacterium damselae* subsp. *piscicida* cells were able to bind protoporphyrin IX and biotinylated bovine haemoglobin (results not shown). Competitive binding assays were performed with strains DI 21, B 51 and ATCC 29690 cultured under iron-limited conditions to investigate whether different haem compounds shared the same binding site or receptor. Similar results were obtained for all these strains and Fig. 4 shows the results for strain DI 21. No inhibition in the binding of haemin was observed when cells of virulent and non-virulent strains were pre-incubated with haemoglobin. Although a marked decrease in the ability to bind haemin was detected in some assays when protoporphyrin IX was used as a competitor, the differences were not statistically significant (p > 0.05).

## DISCUSSION

Haem compounds are known to be an important iron source for many pathogenic bacteria. It has been reported that *Photobacterium damselae* subsp. *piscicida* is able to utilise haemin and haemoglobin as the sole iron source *in vitro* (Magariños et al. 1994).

The present results of the haemin binding assays carried out by the 2 methods described show that whole cells of the 4 strains of *Photobacterium damselae* subsp. *piscicida* tested were able to bind the compound. The binding was significantly decreased by pre-treatment of bacterial cells with Pro-
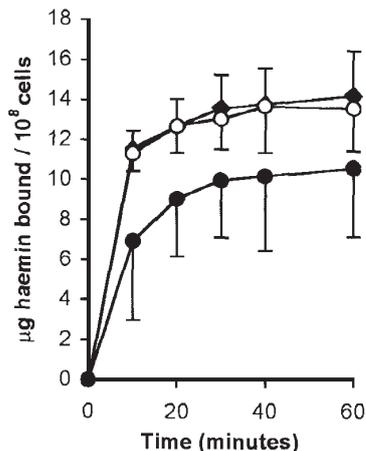
Fig. 4. Competitive binding between protoporphyrin IX or haemoglobin and haemin by *Photobacterium damselae* subsp. *piscicida* DI 21 cultured under iron-restricted conditions. Cells were allowed to pre-bind protoporphyrin IX (●) or haemoglobin (○) for 1 h and the haemin binding was then determined. Cells not pre-incubated with the competitors (◆) were used as controls

teinase K, indicating that protein receptors located at the cell surface are involved in the binding of haemin.

It has been suggested that haem acquisition by bacteria might involve a receptor-mediated process with, as an initial step, specific interaction with surface-exposed protein receptors (Lee 1995). Using the dot blot assay, it was found that total membranes, as well as outer membrane proteins, were able to bind haemin. However, for the same amount of protein, stronger binding to the outer membrane proteins than to total membrane protein extracts was seen, indicating that a higher density of haemin receptors is present in the outer membrane fraction. The binding of haemin by *Photobacterium damselae* subsp. *piscicida* membrane protein extracts was not affected by heat treatment but was almost completely abolished by Proteinase K treatment. These results suggest that cell surface-associated, heat-resistant proteins are mainly responsible for the binding of haemin by *P. damselae* subsp. *piscicida*.

The occurrence of a residual haemin binding observed after Proteinase K treatment of *Photobacterium damselae* subsp. *piscicida* whole cells suggests that, in addition to protein-receptors, some other, protease-resistant, component(s) could also bind haemin. Using the dot blot assay, it was shown that *P. damselae* subsp. *piscicida* capsular polysaccharide was able to bind haemin but with a much lower affinity than the binding by membrane proteins. This suggests that capsular polysaccharide could have contributed to the haemin binding reported in this study. We cannot rule out the possibility that some other protease resistant bacterial components, such as lipopolysaccharide, may participate in the haemin binding of *P. damselae* subsp. *pisci-*

*cida*, as has been reported for other bacterial species (Grenier et al. 1997).

Some bacterial pathogens, such as *Vibrio anguillarum*, *V. vulnificus*, *Photobacterium damselae* subsp. *damselae* and *Aeromonas salmonicida*, possess surface receptors for haemin and haemoglobin, the expression of which is not regulated by iron (Fouz et al. 1994, Hirst et al. 1994, Fouz et al. 1996, Mazoy & Lemos 1996, Mazoy et al. 1996). In contrast, other microorganisms, such as *Neisseria gonorrhoeae*, *N. meningitidis*, *Haemophilus influenzae* and *Yersinia enterocolitica*, express iron-regulated haemin receptors (Lee 1992a,b, 1994, Stojiljkovic & Hantke 1992). In the present study, using 2 different techniques, it was found that the binding of haemin by whole cells of the *P. damselae* subsp. *piscicida* virulent strains DI 21 and B 51 and non-virulent strain ATCC 29690 cells was greatly increased under iron-limited conditions. In contrast, iron limitation did not significantly enhance the binding of haemin by the non-pathogenic EPOY 8803-II cells. These differences were not due to differences in viability because colony forming units (CFU) counts of virulent and avirulent strains cultured with or without added iron were not different (data not shown). Instead, those results could be due to the existence, in all the strains tested, of constitutive receptors for haemin and additional inducible haemin receptors in DI 21, B 51 and ATCC 29690 but not in the EPOY 8803-II cells. However, for reasons not yet understood, no differences were detected when binding of haemin by membrane protein extracts from iron-limited or iron-supplemented bacteria was compared. One possible explanation for this finding is that the inducible receptors, but not the constitutive ones, are sensitive to the sonication treatment used in the membrane isolation protocol.

The presence of efficient iron uptake systems has been considered an important virulence determinant of several bacterial pathogens (Payne 1993). Both *Photobacterium damselae* subsp. *piscicida* virulent strains tested in this study were able to increase the binding of haemin in response to iron limitation. The non-pathogenic strain ATCC 29690 behaved like the virulent ones with respect to haemin binding, while the other non-virulent strain (EPOY 8803-II) was not able to increase the binding of haemin when cultured under iron-limited conditions. These results are not surprising, as virulence almost never results from the presence of only one component and/or mechanism, being rather a multifactorial process. It was previously reported that the amount of capsular polysaccharide expressed by *P. damselae* subsp. *piscicida* cells is dependent on iron availability, being higher in iron-supplemented cells than in iron-limited ones (Magariños et al. 1996a, do Vale et al. 2001). This observation suggests that the *P. damselae* subsp. *piscicida* capsular

polysaccharide would play a minor role in the binding of haemin. The results of the present study, showing that the virulent and constitutively capsulated strain DI 21 (Magariños et al. 1996a, do Vale et al. 2001) and the non-virulent and non-capsulated strain ATCC 29690 (Magariños et al. 1996a) behaved very similarly with respect to the binding of haemin, also support this suggestion. Surface outer membrane protein receptors would therefore be responsible for most of the haemin-binding ability of these bacteria. Such binding may be favoured by a reduction in the amount of capsular polysaccharide.

The results of the competitive binding studies suggest that binding of haemin and protoporphyrin IX, but not of haemoglobin, by *Photobacterium damselae* subsp. *piscicida* cells is mediated by some common receptor(s), although the existence of additional specific receptors for each of the compounds cannot be excluded.

In conclusion, the present work shows that constitutive as well as inducible mechanisms of haemin binding occur in *Photobacterium damselae* subsp. *piscicida*. These mechanisms would rely mainly upon the direct interaction between the haemin molecules and surface-exposed outer membrane protein receptors. Studies on the characterisation of proteins or genes involved in the uptake of haemin by *P. damselae* subsp. *piscicida* are in progress.

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