Identification of heme-binding proteins in the cell membranes of
*Vibrio anguillarum*

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Abstract

Two strains of *Vibrio anguillarum* belonging to O1 and O2 serotypes were examined for their ability to bind hemin and hemoglobin. Whole cells as well as membrane extracts from both strains could clearly bind hemin and hemoglobin constitutively. Hemoglobin binding was completely inhibited by a 100-fold excess of unlabelled hemoglobin and, also by hemin, suggesting the existence of specific receptors for heme groups in the cell membranes. Several hemin-binding and hemoglobin-binding bands with similar molecular sizes were detected in polyacrylamide gels as well as in Western blots. Only two of these protein bands in both strains were iron-regulated while the others were independent of the cell iron status. We conclude that both serotypes of *V. anguillarum* possess heme-binding abilities by means of membrane proteins.

Keywords: *Vibrio anguillarum*; Iron uptake; Hemin-binding; Hemoglobin-binding

1. Introduction

The majority of iron in vertebrates is intracellular, contained in ferritin, hemosiderin or heme, and that which is extracellular in serum or other body fluids is tightly bound to lactoferrin or transferrin. To survive into the host, pathogenic bacteria have developed sophisticated mechanisms to scavenge this essential element for their growth. One of these systems involves the synthesis of siderophores that bind extracellular iron. Ferrisiderophore internalization is achieved by specific receptors, mainly proteins located at the cell surface and associated with other periplasmic and membrane proteins [1]. Other bacteria can use heme-containing compounds as the only iron sources. In this case, acquisition of iron from heme may be facilitated by the production of hemolysins or cytotoxins which can lyse host cells and release intracellular iron [2].

*V. anguillarum* is one of the most important fish pathogens and causes the disease known as vibriosis, a terminal hemorrhagic septicemia. This microorganism produces, under iron-restricted conditions, siderophores and specific receptors for the iron-siderophore complexes [3]. *V. anguillarum* can also use iron from heme compounds by a mechanism not yet understood [4]. In the present work we report hemin and hemoglobin binding activities in *V. anguillarum* cells and identify some cell envelope proteins able to specifically bind heme groups.

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2. Materials and methods

2.1. Bacterial strains and growth media

_V. anguillarum_ strains H775-3 (a plasmidless siderophore-deficient derivative of strain 775), belonging to serotype O1 and RV22 (serotype O2), and _E. coli_ HB101 have been previously described [3,4]. _V. anguillarum_ strains were grown aerobically at 25°C, shaken at 170 rpm in flasks containing M9 minimal medium supplemented with 0.2% (wt/vol) Casamino Acids (CM9). _E. coli_ HB101 was grown in CM9 with shaking at 37°C. All glassware and medium components were deferrated by treatment with 8-hydroxyquinoline (1% in chloroform). The iron content of CM9 medium was always lower than 1 μM, which is sufficiently low to induce iron-regulated proteins in _V. anguillarum_ [3]. Iron-replete medium was achieved by adding 50 μM ferric chloride to CM9.

2.2. Hemoglobin binding assays

Bacterial cells, grown in CM9 medium and in CM9 with 50 μM ferric chloride, were harvested by centrifugation, washed and resuspended in CM9. The cells were filtered onto nitrocellulose paper and tested for biotinylated hemoglobin-binding by a dot blot assay as previously described [5]. Hemoglobin (bovine) was purchased from Sigma and labeled with biotin (Boehringer Mannheim) using the protocol of Lee and Hill [6]. Specific binding was defined as the difference between labeled hemoglobin bound in the presence and absence of 100-fold concentration of non-labeled hemoglobin [6]. The effect of proteases on binding activities was evaluated by incubating whole bacterial cells (prior to dot blotting) in PBS buffer pH 7.5 containing trypsin (1 mg ml⁻¹) or proteinase K (0.2 mg ml⁻¹) (Boehringer) for 90 min at 37°C. The cells were washed twice with CM9, resuspended in the same medium and filtered (20 μl) onto nitrocellulose paper. Hemoglobin binding was then assayed as above.

Hemoglobin-binding was also assayed in cell membrane extracts by the dot blot method. Whole cell envelopes of strains H775-3 and RV22, obtained as above were also assayed by the dot blot method for hemin-binding.

Hemin-binding proteins were also detected in polyacrylamide gels. Samples of whole and outer membrane proteins were diluted 1:1 in a Final Sample Buffer (FSB) (65 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromphenol blue), boiled for 10 min and allowed to cool to room temperature. Then 3 μl of 10 mM hemin were added to 20 μl of protein sample. The mixture was incubated for 1 h at room temperature and electrophoresed as above.

After electrophoresis the gel was incubated in 12.5% trichloroacetic acid (TCA) for 30 min, followed by a 30 min water wash. Hemin binding bands were visualized using the DMB staining procedure described above. Thereafter the gel was stained with Coomassie blue.

2.3. Hemin-binding assays

The assay was performed as described above for hemoglobin, but the cells were incubated with 10 μM (final concentration) bovine hemin (Sigma) for 5 h at 30°C. Although hemin binding was easily visible we used an heme-specific stain [7] to increase contrast. Briefly, after incubation with hemin, the paper was washed with water and stained with dimethoxybenzidine (DMB). The stain was prepared by dissolving 100 mg of DMB (Sigma) in 90 ml of distilled water and adding, immediately before use, 10 ml of 0.5 M sodium citrate buffer (pH 4.4) with 0.2 ml of 30% H₂O₂. The nitrocellulose was allowed to stain in this solution for several seconds, washed with water and photographed.

Whole cell envelopes of strains H775-3 and RV22 obtained as above were also assayed by the dot blot method for hemin-binding.

Whole and outer membrane proteins, obtained as previously described [3], were analysed by SDS-PAGE. Samples (20 μl) were diluted 1:1 with FSB and boiled for 10 min. Gels were made with 3.5% acrylamide in the stacking gel and 12.5% acrylamide in the separating gel and were run at 35 mA for 5 h. After electrophoresis, proteins were transferred to nitrocellulose paper (0.22 μm pore size, Sigma) at
150 mA overnight in 25 mM Tris, 192 mM glycine and 20% methanol. Hemin or hemoglobin-binding bands were identified in the Western blots using the protocols described above for whole cells binding assays.

3. Results and discussion

3.1. Hemoglobin-binding by cells and membranes

V. anguillarum strains H775-3 and RV22 were assayed for hemoglobin-binding activities. Using a dot-blot assay, the cells from both strains bound biotinylated hemoglobin with a strong signal proportional to the number of cells filtered onto the nitrocellulose paper (Fig. 1a). However, no difference in hemoglobin binding was observed between cells grown in iron replete and iron depleted conditions (Fig. 1a), suggesting that these strains express hemoglobin binding activities constitutively. Similar findings were reported for hemoglobin-binding in Vibrio damsela [5] and Aeromonas salmonicida [8]. Binding controls using E. coli cells (Fig. 1a) and assays in absence of hemoglobin were always negative. Furthermore, biotinylated hemoglobin binding in the presence of 100-fold non-biotinylated hemoglobin was negligible, which indicates that the observed labeled-hemoglobin binding was specific. Moreover, hemoglobin-binding was also inhibited when immobilized cells were previously incubated with 10 μM hemin (data not shown). The ability of hemin to inhibit the binding of biotinylated hemoglobin competitively, as has been reported for N. meningitidis [6], suggests that the ligand recognized by the receptor(s) is the heme molecule. In contrast, in Haemophilus influenzae heme itself does not seem to be directly responsible for binding [9].

When bacterial cells were preincubated, prior to dot blotting, with trypsin or proteinase K, a marked decrease in the binding signal was observed, suggesting that one or more outer membrane proteins are directly involved in the binding of hemoglobin by bacterial cells. The fact that we have not found any hemoglobin degradation activities in cell-free supernatants of these V. anguillarum strains (unpublished data) supports the hypothesis that a direct interaction hemoglobin-cell envelope must be necessary for the utilization of heme as iron source [4].

Essentially identical results were obtained when total crude membranes were used instead of whole cells. These membrane extracts from H775-3 and RV22 showed specific binding of biotinylated hemoglobin. Again, no difference was seen between total crude membranes of cells grown with or without added iron to CM9 medium (Fig. 1b). The binding of biotinylated hemoglobin to membrane extracts was also specifically inhibited by competition with 10 μM hemin.

3.2. Hemin-binding by cells and membranes

When V. anguillarum cells from strains RV22 and H775-3 were filtered onto nitrocellulose paper

![Fig. 1. Biotinylated hemoglobin-binding by cells (a) and membranes (b) of Vibrio anguillarum strains H775-3 (A) and RV22 (B). Subscript 1: cells grown in CM9 medium without added iron. Subscript 2: cells grown in CM9 supplemented with 50 μM ferric chloride. 1x = 2 x 10⁷ cfu; E = E. coli HB101.](https://academic.oup.com/femsle/article-abstract/135/2-3/265/572324)
and were assayed for their hemin-binding activities, both strains showed a strong binding of hemin, independently of the iron content of the medium and dependent of the number of cells filtered onto the paper (data not shown). No hemin binding was observed in *E. coli* strain HB101 used as control. Identical results were obtained with total crude membranes from both strains. The results suggest that hemin binding in RV22 and H775-3 is independent of the iron levels in the growth medium.

### 3.3. Identification of hemin-binding proteins

Hemin-binding protein bands were identified in polyacrylamide gels after electrophoresis by means of a specific heme stain containing DMB. Since the sample buffer contained 2-mercaptoethanol some bands, although visible, appeared faint after staining with DMB, resulting in photographs of poor contrast. This is likely due to the release of heme from the protein by the reducing agent when the binding is not covalent. However the presence in a band of oxidized DMB increases staining by Coomassie blue [7], so the bands were first stained with DMB, marked in the gel and then Coomassie blue stained to increase the contrast (Fig. 2). The molecular masses of the bands from cells grown in CM9...
without added iron, were 97, 79, 76, 56, 46, 39 and 29.5 kDa in H775-3 (Fig. 2a) and 88, 82, 76, 46, 37 and 29.5 kDa in RV22 (Fig. 2b). When protein samples were not preincubated with hemin, no DMB stained bands were visualized in any of the strains tested. No differences were seen between total and outer membrane protein extracts, although some heme binding protein could exist that is not detected by this procedure.

When separated proteins were transferred to nitrocellulose and probed with hemin the bands observed were the same as detected in the gel shown in Fig. 2. The high number of hemin binding proteins observed in H775-3 and RV22 is not unique. In Porphyromonas gingivalis several heme binding bands have been also described when this microorganism was grown in heme deplete [10] or heme replete [11] conditions.

3.4. Identification of hemoglobin-binding proteins

Whole and outer membrane proteins from V. anguillarum cells grown in CM9 or in CM9 with 50 µM ferric chloride were separated by SDS-PAGE, transferred onto nitrocellulose membranes and probed with biotinylated hemoglobin for their hemoglobin binding activities. Hemoglobin binding bands of 97, 79, 76, 56, 46 and 39 kDa were detected in whole membrane proteins as well as in outer membrane proteins from H775-3 (Fig. 3). In strain RV22 bands of 88, 82, 76, 46 and 37 kDa were detected in outer and whole membranes (Fig. 3). Moreover, in some experiments, a band of 29.5 kDa was visible in both strains. Also a band of 63 kDa was detected in both strains, which was not present in outer membrane preparations, suggesting that it might be located in the inner membrane.

In contrast to other microorganisms, in which the heme binding bands are only visible under hemin or iron restricted conditions [12,13], only two of the heme binding bands detected in V. anguillarum (with molecular sizes of 79 and 76 kDa in H775-3 and of 88 and 76 kDa in RV22) are iron regulated, because they were not detected in samples from cells grown in iron replete conditions (Fig. 3, 1). All other bands bound hemin or hemoglobin strongly and independently of the iron contents of the growth medium. This may explain why in the dot-blot assays the binding by cells or whole membranes was the same under iron deplete or iron replete conditions.

Hemoglobin-binding bands and heme-binding bands had essentially the same molecular size, strongly suggesting that the heme molecule must be the ligand recognized by the proteins. Nevertheless a protein of 63 kDa, possibly located in the inner membrane of both strains, appears to react in Western blots only with the biotinylated hemoglobin, although this could be due to a poor resolution of the heme staining.

4. Conclusion

In conclusion, we have demonstrated the presence of heme-binding capacities and identified heme-binding proteins in the cell surface of V. anguillarum strains from serotypes O1 and O2. Work is in progress to further characterize the components of this system for acquiring iron from heme.

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