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The *frpB1* gene of *Helicobacter pylori* is regulated by iron and encodes a membrane protein capable of binding haem and haemoglobin

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FrpB1 is a novel membrane protein of *Helicobacter pylori* that is capable of binding both haem and haemoglobin but consistently shows more affinity for haem. The mRNA levels of *frpB1* were repressed by iron and lightly modulated by haem or haemoglobin. The overexpression of the *frpB1* gene supported cellular growth when haem or haemoglobin were supplied as the only iron source. Three-dimensional modelling revealed the presence of motifs necessary to bind either haem or haemoglobin. Our overall results support the idea that FrpB1 is a membrane protein of *H. pylori* that allows this pathogen to survive in the human stomach.

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1. Introduction

Haem is an available iron source in humans and is sequestered by proteins, such as haemoglobin (Hb) or haemopexin. Hb is potentially abundant in humans and forms the majority of the circulating haem supply [1]. Therefore, such sources are attractive to pathogens because they can access them in many tissues during infection to meet their iron needs [2,3]. To use haem or Hb, bacterial pathogens have developed sophisticated mechanisms to obtain iron from these sources, which consist of secreted soluble proteins named haemophores that transport the iron source to a membrane receptor [4]. The iron source is then introduced into the bacterial cytoplasm via a Ton B-dependent mechanism [5]. It has been proposed that all these proteins bind the iron source via two motifs (FRAP and NPNL) [6]. Although these receptor proteins have been identified in several bacteria, including *Escherichia coli* ChuA (69.5 kDa) [7] or *Corynebacterium diphtheria* (24.1 kDa) [8], there are bacteria that possess more than one receptor. For instance, *Pseudomonas aeruginosa* expresses PhuR and HasR, and both receptors are capable of binding haem and Hb [9]. In addition, *Helicobacter pylori* expresses three haem-binding outer membrane proteins, with sizes of approximately 48, 50 and 77 kDa, but their respective identities remain unknown [10]. Recently, another protein termed FrpB2 (90.8 kDa) was identified in *H. pylori*. This protein binds Hb and is located in the membrane [11]. In addition, microarrays assays showed that under iron starvation resulted in a 10-fold increase, the transcript levels of gene, *frpB1* encoding a hypothetical iron-regulated outer membrane protein FrpB1. However, its function remains undetermined [12]. As there is no evidence that *H. pylori* secretes haemophores to scavenge haem or Hb and deliver it to a membrane receptor, it is tempting to speculate that this pathogen must be equipped with more membrane proteins that directly bind these iron sources, as proposed by Worst et al. [13]. However, it is not feasible to study these genes of *H. pylori* by mutagenesis, because a deletion could be easily compensated for by another gene or pathway, such as in *Haemophilus influenzae* [13]. Accordingly, we present a strategy that allows us to suggest that FrpB1 is a new haem- and Hb-binding protein with characteristics different from those reported previously for other proteins. It is possible that the FrpB1 protein participates in a more sophisticated mechanism that has developed in *H. pylori*, which is necessary to confront the extremely acidic environment of the stomach.

2. Materials and methods

2.1. Quantification of frpB1 mRNA levels

Total RNA of *H. pylori* growing under conditions: iron replete, iron absence (medium was supplemented with 2,2′-dipyridyl),

Abbreviations: csp, cold shock protein; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; K_d, dissociation constant; NaH_2PO_4, monobasic sodium phosphate; PBS, phosphate-buffered saline; SD, standard deviation; UV/VIS, ultraviolet/visible

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and haem or Hb used as the sole iron source was purified using the phenol/chloroform technique [14]. Semi-quantitative RT-PCR was performed using 10 ng of total RNA, SuperScript One-Step (Cat. 11922028, Invitrogen) and specific oligonucleotides for *frpB1* (forward, 5′-ACTAAAGGCGCGGCGAATGCGAGCGC-3′, and reverse, 5′-GGACACCCTTGAGCGCCATCAGGGCT-3′). The primers were designed according to the annotated sequence in PubMed (HP0876). The RT-PCR conditions for the *frpB1* gene were as follows: 45 °C for 30 min, 94 °C for 2 min, 23 cycles (at 94 °C for 1 min, 50 °C for 1.30 min, and 72 °C for 1.20 min), and a final extension step at 72 °C for 10 min. As a positive control, a reaction using oligonucleotides that amplify 235 bp of the 23s mRNA was performed. A reaction was prepared without reverse transcriptase as a negative control. The amplicons were separated on 1% agarose gels, which were stained with ethidium bromide and subjected to densitometric analysis using Quantity One-4.6.3 1-D Analysis Software. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, (www.graphpad.com).

2.2. Cloning of the frpB1 and chuA genes

The *frpB1* gene was amplified from *H. pylori* genomic DNA by PCR using the primers, 5′-GGGGGGAGCTCATGTTTTTAAGATCATACCCAAAGCTTAGATA-3′ (forward) and 5′-GGGGGGAATTCCTACCATTTGTAAGCCACTTCAAAACG-3′ (reverse), designed according to annotated sequences (GeneID NCBI accession number 890085). The *chuA* gene used in this work as a positive control was amplified from *E. coli* (strain *EC EH O157:H7 EDL 933*) genomic DNA using the primers, 5′-GGGGGGAGCTCATGTCACGTCCGCAATTTACCTCGTTGCGTTTGAGTTT-3′ (forward) and 5′- GGGGGGAATTCTTACCATTGATAACTCACGAAAATTTTTCCGTTACGACCATC-3′ (reverse) (GeneID NCBI accession number AAG58641), designed according to an in silico analysis. Both of the PCR products were inserted in pColdI DNA (Cold-Shock expression vector, Takara) using the *Sac*I and *Eco*RI restriction sites. The identity of each gene was corroborated by sequencing analysis.

2.3. Expression of FrpB1 and ChuA proteins in E. coli

E. coli (BL21pLys) competent cells transformed with pCold-frpB1 or pCold-chuA were cultivated in Luria–Bertani medium to 0.4 O.D. Gene expression was induced by adding 1 mM IPTG, and the culture was incubated at 30 °C. The cells were then collected by centrifugation (8000×g), and the pellet was suspended in PBS plus lysozyme (1 mg/ml). Aliquots of 1 mM PMSF and Triton X-100 were added to a final concentration of 1%. The cells were broken by sonication at 4 °C for 2 min, using 20-s pulses and 60% amplitude. The samples were then centrifuged at 1500 X g for 5 min. The supernatant was ultracentrifuged at 105 000×g for 1 h to obtain soluble and insoluble fractions. The insoluble fraction, considered as a source of membrane proteins, was suspended in PBS containing 1 mM PMSF and 1% Triton X-100. The samples were then loaded onto a Nickel-Sepharose High Performance column (Qiagen). The flow-through fraction was collected, and non-specific interactions were eliminated by washing the resin three times with wash buffer (containing 50 mM NaH_2PO_4 and 300 mM NaCl) and 50, 65, and 80 mM imidazole. The proteins were released with pH 8.0 elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole), and each fraction was analysed using Coomassie blue-stained SDS–PAGE. The presence of the FrpB1 and ChuA proteins was corroborated using Western blot analysis using anti-His antibodies.

2.4. Overlay assay

The purified proteins (FrpB1 and ChuA) separated by 10% SDS–PAGE were transferred to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat milk in PBST buffer (PBS, pH 6.8, and 0.05% Tween 20). Hb (10 mM iron) was added and incubated for 12 h at 4 °C. After three washes (15 min) with PBST, the membrane was incubated with anti-β-globin antibodies. Lastly, the secondary antibody coupled to horseradish peroxidase was added, and the signal was developed using chemiluminescence (Millipore) [15].

2.5. Haem-binding assay

Binding assays were performed to test the ability of the FrpB1 protein to bind haem. Purified protein (20 μM) was suspended in binding buffer (250 mM Tris, 5 mM EDTA, 10% glycerol and 20 μM haem, pH 8.0), and the samples were incubated at 37 °C for 30 min and loaded onto 7% native PAGE. Proteins were visualised by Coomassie blue-staining, and the haem interaction was revealed by the hydrogen peroxide technique (a brown colour is observed in the gel) [16].

2.6. Haem and Hb assays to estimate the binding affinity

To estimate the affinity of the FrpB1 and ChuA proteins for haem or Hb, binding assays were performed using UV–VIS spectrophotometry. Purified proteins (20 μM) in binding buffer were titrated with increasing concentrations of haem or Hb (1, 2, 7, 15, 46, 77, 150, 310, 460, 610, 770, and 930 μM). The absorbance in the UV–VIS spectrum between 200 and 900 nm was scanned using a spectrophotometer (Supplementary data Figs. 10 and 11, supplementary material). The spectra were recorded 1 min after the addition of each haem or Hb aliquot in triplicate in three separate experiments. The intensities of the Soret peaks at 238 nm of the haem-FrpB1 (Fig. 10B), at 451 nm of Hb-FrpB1 (Fig. 10C), at 245 nm of haem-ChuA (Fig. 11B) and at 456 nm of Hb-ChuA (Fig. 11C) were evaluated to monitor the complex formation. To generate the binding isotherms, the absorbance values were plotted against the haem or Hb molar concentration (Supplementary data). The data were fitted to a one binding-site model using the non-linear regression function to determine the dissociation constant (K_d), assuming that binding follows the law of mass action (Supplementary data). Statistical analyses, binding stoichiometry and affinity estimations were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, (www.graphpad.com).

2.7. Media and cultures

It has been documented previously that ingredients in the culture medium, including peptone or tryptone, can block the uptake of Hb or haem. For this reason, the cells were cultured in M63 minimal medium supplemented with ferric citrate [17]. *E. coli* transformed with the pCold, pCold-frpB1 or pCold-chuA plasmids were inoculated on plates containing 250 μM 2,2′-dipyridyl (Sigma–Aldrich), 1 mM IPTG (Fermentas, Life Sciences) or supplemented with equivalent molar quantities (10 mM iron) of haem or Hb (Table 1) and incubated at 30 °C for 28 h. In the case in which growth cell was observed, the diameter of colonies was measured (diameters oscillating between 2 and 3 mm were observed). Positive cellular growth was indicated as (✓) and negative (×) in which no colonies were observed.

3. Results

3.1. The expression of the frpB1 gene is increased in H. pylori under iron absence conditions and slightly modulated by the supply of haem or Hb

It is known that the mRNA levels of the *frpB1* gene are increased under iron-limiting conditions [12]; therefore, other iron sources,

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