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Research Article

Identification and Characterization of a Family of Outer Membrane Proteins of *Helicobacter pylori*, which Scavenge Iron from Human Sources

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ABSTRACT

Introduction: *Helicobacter pylori* is a gram-negative spiral bacterial, it has been associated with peptic ulcers, gastritis, duodenitis, and it is believed to be the causative agent of gastric cancer. The sources such as human lactoferrin, haem and haemoglobin can support the *H. pylori* growth. However, is still not fully understood how the process of iron acquisition occurs. An in silico analysis has shown that *H. pylori* genome has a family of three outer membrane proteins regulated by iron (FrpB). Two of them: FrpB1 and FrpB2 were purified as recombinant proteins and their haem- or haemoglobin-binding capability was demonstrated. Unfortunately, the last protein of the family (FrpB3) has not been investigated.

Methods: In this work FrpB3 was purified by haem-affinity chromatography and its capacity of haem-binding was analyzed. This protein was identified by mass spectrometry and its expression was quantified by real time technique under different human iron sources. This expression was compared with FrpB1 and FrpB2. The FrpB3 structure was analyzed by 3D model to view the motifs necessary for Hb-binding, and also was compared with FrpB1 and FrpB2 structures.

Results: The protein identified was FrpB3, its respective gene was overexpressed with haemoglobin. FrpB1 was overexpressed with haem, while FrpB2 was induced in presence of haem and also haemoglobin. Both 3D models showed that they are structurally conserved because they have the typical barrel structure, which is inserted in membrane, also, the motifs necessary for Hb-binding were identified in all the structures.

Conclusion: *H. pylori* expresses FrpB1, FrpB2 and FrpB3 proteins to scavenge iron and they are regulated according to availability of iron source, maybe in order to withstand the extreme environment present in the stomach. Our overall results represent the effort to explain the importance of iron acquisition.

Keywords: *Helicobacter pylori*; Iron acquisition; Haemoglobin; Haem; Starvation; Overexpression

INTRODUCTION

Helicobacter pylori is a gram-negative bacterium belonging to genus epsilon bacteria [1,2], measuring 2 to 4 microns long and 0.5 to 1 micron wide, morphologically it can be observed like flagellated bacillus or coccus. It grows up in a microaerophilic environment [3]. *H. pylori* infects approximately 50% of the world population [4], having a higher incidence in underdeveloped countries [5] and affecting the early childhood population [6]. There is a relationship between infection and hygiene. *H. pylori* colonizes the stomach without causing disease. However, it should be considered as a risk factor for the development of several clinical disorders in the gastrointestinal tract such as acute and chronic gastritis, peptic ulcer, dyspepsia and about 2% could develop gastric cancer [2].

All human pathogens bacteria require iron for their metabolism and survival [7]. They need iron to catalyze several metabolic processes such as respiratory chain [8] and infectious processes [9]. However, in the human, free iron source is insufficient for

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bacteria, therefore, they have developed mechanisms to acquire iron from human sources [10]. *H. pylori* habits an environment, where the availability of iron is limited [11], and has a large amount of proteins for iron acquisition, for instance, metalloproteins [12,13-16]. This bacterium synthesizes three membrane proteins of 48, 50 and 77 kDa capable of binding haem, unfortunately, their identities remain undetermined [17]. Then, two outer membrane proteins were characterized as Hb-binding proteins: FrpB1 of 88.5 kDa and FrpB2 of 90.8 kDa, FrpB2 also binds haem [18,19]. Interestingly, there is another outer membrane protein termed FrpB3, which has not been investigated. Finally, it has been observed that the presence of *H. pylori* can cause anemia in its host, which indicates its enormous capacity of this bacterium to acquire iron from human sources [20,21].

Due to FrpB1 and FrpB2 belong to a family of 3 proteins and that FrpB3 has not been studied yet, we believe that this family of proteins are tightly ligated to iron acquisition in *H. pylori* and maybe the mechanism involves the regulation of 3 proteins. In this work, FrpB3 was investigated and its relation with FrpB1 and FrpB2 was explored. Here, we are presenting the first insights that attempt to explain the mechanism of iron acquisition when *H. pylori* is cultivated under different iron sources and expresses FrpB family to bind iron.

MATERIALS AND METHODS

Growth conditions

H. pylori J99 (ATCC 700824) strain was routinely grown on casman agar (Becton Dickinson 211106) supplemented with 5% defibrinated sheep blood (Dibico 1600 FC), at 37 °C, under microaerophilic conditions (10% CO_2) for 24 h.

Growth curve

Bacteria grown in casman medium were collected and washed three times with 20 mM Tris HCl buffer, then they were transferred to 10 mL of Brucella medium (Becton Dickinson 296185) until 0.250 of optical density, they were cultivated at 37 °C, under microaerobic conditions (10% CO_2) for 24 h. During this period the optical density was monitored every 2 h and a graph was performed in order to obtain the several phases of the growth. Bacteria were collected by centrifugation at exponential phase. At this time, bacteria were collected and washed in three occasions with 20 mM of Tris HCl buffer pH 7.2, after were resuspended in Brucella broth without iron (-Fe), because iron was chelated with 250 μM of 2, 2'dipyridyl (Aldrich 364177), this suspension of bacteria was divided in three parts to monitor the growth of *H. pylori* under different iron sources. 1) bacteria without iron (-Fe), 2) bacteria without iron and supplemented 3 h after the start of the curve with 10 mM Hb (Sigma H7379) (-Fe +Hb) and 3) bacteria without iron, which were supplemented 3 h after the start of the curve with 10 mM ferric ammonium citrate (Sigma-Aldrich F5879) (+Fe), optical density was monitored for every 2 h.

Membrane protein purification and affinity chromatography coupled to haem

H. pylori grown on casman medium was used for membrane protein purification. The biomass was collected and 3 washes were performed with buffer 20 mM Tris HCl pH 7.2. Bacteria were resuspended in the same buffer and supplemented with 1 mM PMSF and 1% sarkosyl (Sigma L9150). Bacteria were lysed by sonication for 6 mn with pulses of 30 s. To eliminate intact cells, samples were centrifuged at 12000 × g for 20 mn. The supernatant was considered to contain total proteins. To isolate membrane proteins, this supernatant was ultracentrifuged at 105000 × g for 1 h at 4 °C, pellet was considered as membrane proteins. Membrane proteins were resuspended in 20 mM Tris HCl pH 7.2 and 1% sarkosyl. After, membrane proteins were loaded onto haem-affinity chromatography (Sigma H6390) and incubated at 4 °C overnight under agitation. Samples were centrifuged at 1000 g for 1 mn, this supernatant was discarded because it contains the proteins without affinity by haem (flow through). The resin was washed in five occasions with 20 mM Tris buffer pH 7.2 in order to eliminate non-specific binding (washes), finally, the proteins that were bound to haem were eluted with 6M guanidine hydrochloride (Sigma G3272) (eluted proteins). The eluted proteins were cleaned by TCA precipitation (kit precipitation 1632130 Bio-Rad) and the concentration of proteins was estimated by Bradford method at 595 nm (Bio-Rad 5000201), after the proteins were loaded onto 12% SDS-PAGE gels and stained with Coomassie brilliant blue R-250.

Mass spectrometry LC-MS/MS

This procedure was performed to identify the selected band from a SDS-PAGE coomassie stained, each band was excised with a scalpel, washed with distilled water and dried, the band was digested with trypsin, subsequently, the sample was loaded onto the Micromass QTof I equipment, 5 μl of the digested sample was injected into a PepMap C18 column (0.75 μm × 15 cm) and eluted with acetonitrile at a linear gradient of 200 nl/minute, the peptide eluted was introduced to the mass spectrometer through a New Objective PicoTip, which in turn was supported by a New Objective adapter. The conditions of the experiment are: capillary voltage 1.8 kV, voltage cone 32 V, fixed collision energy from 14 eV to 50 eV according to the mass and charge of the ion. The data obtained were searched in the database www.matrixscience.com using the Mascot algorithm (Protein Core Facility, Columbia University Medical Center, http://www.cumc.columbia.edu/dept/protein/).

RNA extraction

H. pylori grown in Brucella broth medium was collected in exponential phase and centrifuged. Pellet was washed in three occasions with buffer 20 mM of Tris HCl pH 7.2 and resuspended in Brucella broth free iron, iron was chelated with 250 μM of 2, 2'dipyridyl (Aldrich 364177), this suspension of bacteria was divided in four parts: 1) Basal condition (casman broth), bacteria were seeded on casman agar plates supplemented with 5% sheep blood. 2) Chelating condition (chelator) was seeded on agar casman with 250 μM of chelator

(2, 2' dipyridyl). 3) condition (Hb) was seeded on casman agar with 250 µM of chelator (2, 2' dipyridyl) and supplemented with 20 mM of Hb. 4) condition (haem) was seeded on casman agar with 250 µM of chelator (2, 2' dipyridyl) and supplemented with 20 mM of haem (Sigma 51280). Bacteria were incubated for 20 h at 37 °C and 10% CO_2. Cellular cultures were collected and 3 washes were performed with buffer pH 7.2, then the RNA was extracted by Trizol method (Ambion 15596018). A q-RT-PCR was performed, using the primers of Table 1. The conditions of the q-RT-PCR were the following: 50 °C for 2 mn, 95 °C for 15 mn, 40 cycles (94 °C for 15 s, 52 °C for 30 s and 72 °C for 30 s). Primers to amplify the sub region of the *16S* gene were used as a positive control, while negative control was prepared without the reverse transcriptase. The quantification of mRNA expression levels of all genes was performed with the delta Ct (2-ΔΔCt) method [22-24] and the results were analyzed with the program GraphPad 8.02.

Table 1: Primers used to quantify mRNA by real-time PCR technique. They were designed from *16S*, *FrpB1*, *2* and *3* genes of *H. pylori*.

Primer	*RNA 16S*	mer	Tm	Amplify
Forward	5´CGGGCTAAACGCGCTAGGCGGC3´	22	52	162
Reverse	5´GCGCTCAAAGACCCTAAATGCAGGGGC3´	27	53	
	FrpB1			
Forward	5´GCAGCGAGTGGGGCGGTGAGTT3´	22	52	219
Reverse	5´GCTCGCTGGGAGTTCCTGGCTC3	22	52	
	FrpB2			
Forward	5´GGGCGGTGGTGCGGTGATAGCG3´	22	52	182
Reverse	5´CGCTCGCTTGAGCCGCCCCC3´	20	52	
	FrpB3			
Forward	5´GGTCCAGGCGCGGTTGCGGG 3´	20	52	150
Reverse	5´CCCCCGATAGGCTGCAGTGGCG3´	22	52	

Amino acid sequences alignment of FrpB1, FrpB2 and FrpB3

Amino acid sequences of FrpB1 (Q9ZKX4), FrpB2 (Q9ZKT4) and FrpB3 (Q9ZJA8) of *H. pylori* were submitted to the ClustalW server https://www.ebi.ac.uk/Tools/msa/muscle/ in order to perform the alignment and the JalWiev 10.2 program was used to highlight the FRAP and NPNL motifs, the ChuA (Q7DB97) sequence of *E. coli* was used as a template.

Modeling in the protein space

Amino acid sequences of the FrpB1, FrpB2 and FrpB3 of *H. pylori* and ChuA of *E. coli* were submitted to the server http://www.cbs.dtu.dk/services/CPHmodels/ , the PDB file was obtained and displayed in the Chimera 1.12 program.

RESULTS

The exponential phase of *H. pylori* started at 6 h and ending to 20 h

H. pylori was cultivated in order to investigate the exponential phase in Brucella medium. Samples were collected each 2 h, and its cellular growth was determined by optical density. The exponential phase was reached at 16 h.

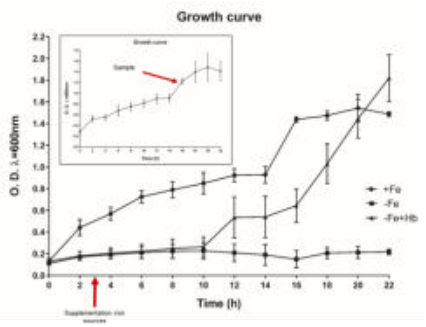

Figure 1: *H. pylori* can support its cellular growth using Hb or free iron as an iron source. *H. pylori* has a normal growth curve when iron is available (box). Samples of *H. pylori* were collected in their exponential phase of cellular growth (box, arrow). Subsequently after 3 h, 10 mM Hb (triangles) and 10 mM ferric chloride (circles) were added to analyze its behavior. A negative control with only chelant (squares) was performed. Cellular cultures were incubated for 22 h and OD was monitored every 2 h, graphs show the standard deviation of three independent biological experiments. For details see Material and Methods section.

H. pylori can support its cellular growth using Hb or free iron as an iron source

In order to investigate if the iron source (Hb or iron) can support the cellular growth of *H. pylori*, bacteria were cultivated under iron starvation.

3 h after, Hb or iron sources were added. Cellular growth was monitored each 2 h. The result showed us that *H. pylori* can support its cellular growth using Hb (triangles) or iron (circles) as only iron source.

However, a negative control (squares) cannot (Figure 1). This result showed us that *H. pylori* has the capacity of supporting its cellular growth using Hb or free iron as only iron source.

FrpB3 had affinity for haem and was identified as iron-regulated outer membrane protein

H. pylori was grown on casman broth for 16 h, then bacteria were collected. Membrane proteins were enriched by ultracentrifugation and purified by haem-affinity chromatography. Samples of each step were loaded onto SDS-PAGE staining with coomassie blue, a band of 97.4 kDa was observed, which corresponds to expected size. The identity of this protein was corroborated by mass spectrometry (Figure 2).

Interestingly, 97.40 kDa band was identified as FrpB3 protein, which belongs to a family of three proteins iron regulated (FrpB) [12]. FrpB1 and FrpB2 were previously characterized by us and were identified as Hb-binding proteins; in addition FrpB2 bound haem too. In the present work the last protein of the family (FrpB3) was characterized as haem-binding protein too (Table 2).

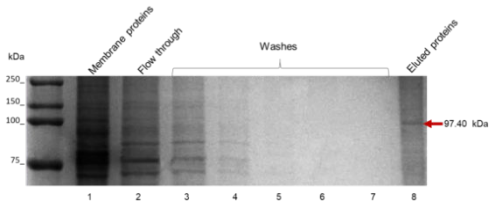

Figure 2: Protein purification by haem-affinity chromatography. Samples were loaded onto haem-affinity chromatography, and then samples of each fraction were collected and analyzed by SDS-PAGE and stained with coomassie blue. Total membrane proteins (lane 1), flow through (lane 2), washes (lanes 3 to 7) and eluted proteins (lane 8). Molecular weight marker is indicated on the left. The estimated size for purified membrane is protein-binding indicated on the right side with arrow.

Table 2: A 97.49 kDa protein of *H. pylori* was identified by mass spectrometry. Search parameters were as follows: type of search, MS/MS ion search; enzyme, trypsin; fixed modifications, carbamidomethyl (C); variable modifications, acetyl (N-term) and oxidation (M); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ± 1.2 Da; fragment mass tolerance, ± 0.6 Da; maximum missed cleavages, 2; instrument type, default; number of queries, 101; and database, SwissProt. The description given in "Fuction" section is the information obtained from the accession number when it is introduced in the UniProt server.

Accession number UniProt	Protein name	Coverage	Mass (kDa/pI)	Mascot score	Unique peptide	Function
Q9ZJA8	FrpB3	16%	97.49/9.05	694	19	Putative iron-regulated outer membrane protein

mRNA levels of *frpB3* gene were increased when haem was supplied as only iron source

To investigate whether the iron source regulates the mRNA expression of *frpB* genes differentially, we perform experiments with *H. pylori*, which was cultivated under iron starvation and the casman broth was supplemented with Hb, haem or iron.

Then mRNA was purified in order to quantify the levels of *frpB* genes. Interestingly, *frpB1* was increased when Hb was added as an iron source (Figure 3A), *frpB2* was increased with haem (Figure 3B) and *frpB3* was increased with haem as only iron source (Figure 3C).

This result clearly showed us that the iron sources can regulate the gene expression of each *frpB* gene differentially.

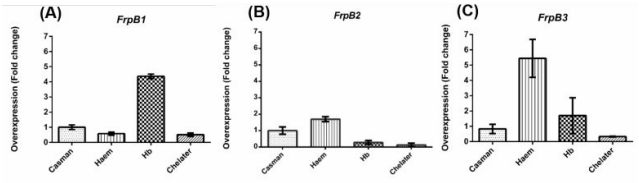

Figure 3: (A-C) *H. pylori* expressed *frpB1*, *frpB2* and *frpB3* genes differentially when the culture media was supplemented with haem, Hb or under iron starvation. The mRNA samples were quantified using real-time PCR. Error bars are showed in order to indicate the dispersion of the data. For details see Material and Methods section.

3D-structure of FrpB family showed the motifs necessary for Hb-binding

Amino acid sequence of FrpB was modeled by Chimera 1.12 program. 3D structure revealed the typical barrel structure (white structure), in black FRAP and NPNL motifs are showed, those are necessary for haem- or Hb-binding (Figure 4). We can see the ChuA protein of *E. coli* has same structure and its motifs FRAP and NPNL.

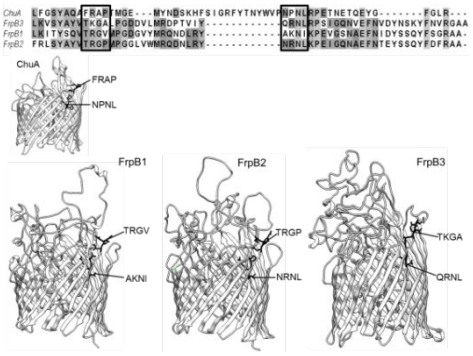

Figure 4: Amino acids alignment and 3D structure modeling of the FrpB proteins. A section of the amino acids of the ChuA protein of *E. coli* and the FrpB proteins of *H. pylori* are showed. FRAP and NPNL motifs necessary for haem or Hb-binding are showed in the boxes. 3D structures are showed below. ChuA protein from *E. coli*, FrpB1, 2 and 3 from *H. pylori*, FRAP and NPNL motifs are highlighted in black, these motifs are observed in all the proteins and they are necessary for Hb-binding.

DISCUSSION AND CONCLUSION

H. pylori is a gram negative bacteria. This human pathogen needs iron in order to survive. In the human host there are several iron sources such as Transferrin (Tf), Lactoferrin (Lf), Haemoglobin (Hb), or haem [25]. To obtain iron this pathogen has developed a mechanism consisting on expressing outer membrane proteins that bind Hb or haem [26].

Those proteins bind the iron source directly and several proteins have been investigated, for instance, FrpB is a protein family composed of three proteins: FrpB1, FrpB2 and FrpB3 [27]. FrpB1 and FrpB2 were investigated previously and they bound Hb, in addition, FrpB2 bound haem too. In this work the last FrpB3 protein was characterized and we found that this protein bound haem (Table 3).

We think that genes that encode these FrpB proteins are regulated by the Fur iron system, which acts as a ferrous-dependent transcriptional repressor, because there are bacteria that express protein regulated by this Fur iron system, for instance, PeuA in vibrio [28], pfeR, pvdS, tonB, and fumC in *Pseudomonas aeruginosa* [29], HasA in *Serratia marcescens* [30], TbpA and TbpB in *Neisseria gonorrhoeae* [31].

Table 3: Summary of the main characteristics of the FrpB family of *H. pylori* proteins.

Protein Name	Accession number UniProt	kDa	Localization	Bound	Reference
FrpB1	Q9ZKX4	88.5	Membrane	Hb, Haem	Carrizo-Chávez et al. [18]
FrpB2	Q9ZKT4	90.8	Membrane	Hb	González-López and Olivares-Trejo [19]
FrpB3	Q9ZJA8	97.5	Membrane	Haem	This investigation

Additionally, an in silico analysis using the Ferric uptake regulation protein (P0A9A9 FUR_ECOLI) from *E. coli* was performed and a Ferric uptake regulation protein was found in the proteome of *H. pylori* (Q9ZM26 FUR_HELPJ). Previously, it was demonstrated that *frpB1* gene has the sequence Fur, which is Fur system repressed, unfortunately, in this investigation the *frpB3* gene was not investigated [32]. Therefore, in the present work Fur sequence (TAATAATnATTATTA) upstream of *frpB3* gene was investigated by in silico analysis. Unfortunately, no Fur sequence was found. All overall could explain why the source (Hb, haem or iron starvation) increased the gene expression of *frpB1, frpB2* or *frpB3*, differentially. Interesting, only *frpB1* of three *frpB* genes was Fur regulated, however all 3D structures of FrpB proteins had the typical barrel structure of membrane protein (Figure 4) [33]. This structure has characteristic of proteins such as Has of *Serratia marcescens* [34], CopB of *Moraxella catarrhalis* [35], HmbR of *Neisseria meningitidis* [36] and has the aminoacids necessary to bind Hb or haem. 3D structures of FrpBs proteins revealed FRAP and NPNL motifs, which are necessary for Hb-binding. Those motifs exist in bacteria such as *Photobacterium damselae* [37], HupO of Vibrio fluvialis [38], BhuR of *Bordetella avium* [39], HemR of *Yersinia enterocolitica* [40] and HmuR of *Porphyromonas gingivalis* [41] and ChuA of *Escherichia coli* [42]. FrpB1 and FrpB2 proteins were not purified by haem-affinity chromatography maybe because *H. pylori* was cultivated under iron sufficiency condition. Other condition such as supplementation with Hb or haem could be better to express those FrpB proteins. Additionally, FrpB3 protein had already been founded previously using the same growth conditions of iron sufficiency [43]. On the other hand, when the expression of each gene was analyzed, this value was different for each iron source. These results clearly suggest that expression of *frpB1, 2* and *3* genes is regulated by iron and also each gene is sensitive to Hb or haem. Other human iron sources were not investigated in the present work, however, they could regulate the expression of *frpB* genes too. In this work we are presenting the first insights that attempt to explain how the expression of a family of outer membrane proteins is regulated by the iron source. *H. pylori* is a human pathogen that requires a high concentration of iron because it has been reported that it can cause anemia when it is infecting humans [21,44,45]. This can explain why *H. pylori* has several genes, which express many proteins involved in iron acquisition such as FrpB1, FrpB2 or FrpB3 [27]. Our overall results attempt to explain why *H. pylori*

is a pathogen equipped with mechanisms involved in iron acquisition, maybe iron plays a crucial role when this bacterium invades and infects the stomach and also for supporting the hostile conditions presents in that tissue.

AUTHORS' CONTRIBUTIONS

González-López and Olivares-Trejo designed the experiments. González-López and Sánchez-Cruz performed the experiments. All authors analyzed the data and wrote the paper. Original idea by Olivares-Trejo.

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