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The gene *frpB2* of *Helicobacter pylori* encodes an hemoglobin-binding protein involved in iron acquisition

Marco A. González-López · José J. Olivares-Trejo

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Abstract Human hemoglobin (Hb) is a metalloprotein used by pathogens as a source of iron during invasive process. It can support the *Helicobacter pylori* growth and several proteins are induced during iron starvation. However, the identity of those proteins remains unknown. In this work, by in silico analysis we identified FrpB2 in *H. pylori* genome. This protein was annotated as an iron-regulated outer membrane protein. Multiple amino acid alignment showed the motifs necessary for Hb-binding. We demonstrate the ability of FrpB2 to bind Hb by overlay experiments. In addition, the overexpression of this gene allowed the cell growth in media without free iron but supplemented with Hb. All these results support the idea that *frpB2* is a gene of *H. pylori* involved in iron acquisition when Hb is used as a sole iron source.

Keywords Hb-binding protein · Hemoglobin · *H. pylori* · Iron starvation

Introduction

Iron is essential for all the pathogens infecting mammalian hosts. In human beings iron is found bound to proteins such as lactoferrins or hemoglobins because in free form is highly toxic (De Freitas and Meneghini 2001; Otto et al. 1992). Human pathogens have developed mechanisms to acquire iron from different human sources. One efficient mechanism consists on the expression of proteins capable of binding Hb (hemophores and Hb-receptors) (Wandersman and Stojiljkovic 2000; Genco and Dixon 2001; Ratlif-Griffin and Stojiljkovic 2004). Hemophores are Hb-binding proteins secreted by the cell, which bind extracellular Hb (Baker et al. 2003; Létoffé et al. 1994), while Hb-receptors are proteins localized in outer membrane. Hb-receptors have the ability to bind and transport Hb into the cell through a Ton B-dependent mechanism (Beddek et al. 2004; Ferguson and Deisenhofer 2002; Simpson et al. 2000). Hb-receptors bind Hb using two conserved motifs (FRAP and NPNL), which are often separated by a 10–20 amino acid segment. Multiple amino acid alignment from different bacterial Hb-binding proteins have revealed that there are changes in at least one of these FRAP and NPNL motifs (Liu et al. 2006; Simpson et al. 2000), notwithstanding of that, their Hb-binding properties are not affected (Ferguson et al. 2002; Letoffe et al. 1999; Liu et al. 2006; Perkins-Balding et al. 2003; Rossi et al. 2001).

Helicobacter pylori is the leading cause of peptic ulcers and gastritis, it can survive in several human environments, it requires iron for surviving and it has been observed that iron overload exacerbates the infectious diseases caused by this pathogen

M. A. González-López · J. J. Olivares-Trejo (✉)
Posgrado en Ciencias Genómicas, Universidad Autónoma de la Ciudad de México, San Lorenzo 290, 03100 México, DF, México
e-mail: jouacm@gmail.com

(Velayudhan et al. 2000). Thus, it has been proposed that *H. pylori* can use different iron sources in the human. One of them is holo-lactoferrin where iron acquisition is mediated by a membrane protein (Dhaenens et al. 1997). Another is heme group, at least three proteins have been isolated by heme affinity chromatography, however, their identity still remains unknown (Worst et al. 1995). In addition to previous sources, *H. pylori* is also able to obtain iron from Hb because it can support its cellular growth using Hb as a sole iron source (Worst et al. 1995). Nevertheless, the mechanism to acquire iron from Hb has not been determined. The usage of diverse iron sources may confer *H. pylori* the ability to survive in different host tissues. As consequence, lactoferrin may be used in mucosa whereas Hb may be used by *H. pylori* when it is in contact with blood cells released during ulceration. In the present work, an Hb-binding protein was identified in *H. pylori*. The *frpB2* gene was cloned and expressed in *E. coli* and the ability to acquire iron from Hb was tested. Then, FrpB2 protein expressed was identified as a membrane protein with Hb-binding activity. Our findings suggest that FrpB2 could be responsible for the iron acquisition from Hb in this pathogen.

Materials and methods

Cultures

E. coli DH5α was used for *frpB2* gene cloning and BL21pLysS strain was used for FrpB2 protein expression. All *E. coli* strains were cultured at 37°C in LB media supplemented with 100 mM ampicillin (Sambrook and Russell 2001).

The reference strain *H. pylori* J99 maintained by freezing at −70°C in brucella broth media (Sigma laboratories) containing 50% (vol/vol) glycerol. Cultures were grown on cassman agar supplemented with 5% (vol/vol) horse blood (Difco laboratories) incubated for 3 days at 37°C under microaerophilic conditions.

Growth kinetics

Overnight cultures of *E. coli* were iron starved by adding 250 μM 2, 2′dipyridyl (Sigma laboratories), thus O.D. was adjusted up to 0.4 and the FrpB2 expression was induced for 3 h with 1 mM IPTG (Fermentas Life Sciences), thereafter cultures were supplemented with equivalent molar quantities of (10 mM iron) Hb, different metalloproteins (Sigma laboratories) or ammonium ferric citrate (Sigma laboratories) and incubated during 60 h. Samples were collected every 2 h to follow cell growth by O.D. 600 nm using spectrophotometry.

frpB2 gene cloning and expression

We amplified this gene by PCR assay using as probe *H. pylori* genomic DNA and specific primers (5′ GGGGG GAGCTC ATG AAT GAC AAG CGT 3′ forward) (5′ GGGGG TCTAGA TTA AAA CTT ATA AGA AAT TTC AAA CCT AGC GTT AAA GCC AGG CTC TGC 3′ reverse) designed according to the reported sequence (NCBI access number 890041). The amplified sequence was cloned under T7 promoter control in pJET1.2 Blunt plasmid (Fermentas Life Sciences). The function of *frpB2* gene was tested by transforming *E. coli* (BL21pLysS), this bacterium lacks of genes that encode for Hb-binding proteins. As a positive control, we used *ChuA* gene of enterohemorragic *E. coli EC EH O157:H7* (Torres and Payne 1997). Positive constructs were selected by ampicillin resistance in *E. coli*, the gene identity was corroborated by sequenciation. FrpB2 protein expression was induced for 3 h with 1 mM IPTG in BL21pLysS cells and proteins were analyzed by SDS–PAGE stained with Coomasie blue.

Purification of membranal and cytosolic fractions

BL21pLysS cells were transformed with pJET-frpB2, pJET-ChuA or pJET plasmids and cultivated in 50 ml of LB medium to 0.4 O.D. Cells were iron starved by adding 250 μM 2-2′dipyridyl, thus expression was induced for 3 h with 1 mM IPTG and the cultures were supplemented with Hb or iron. Cultures were followed for 25 h and the cells were collected by centrifugation. The pellet was suspended in PBS (pH 7.3 cold). PMSF 1 mM and cells were broken by French pressure using 1,500 psi. Samples were centrifuged at 1,500×*g* for 5 min to eliminate unbroken cells and debris. Supernatant was ultracentrifuged at 105,000×*g* for 1 h to obtain soluble and unsoluble fractions. Samples of each fraction were taken and load onto 12% SDS–PAGE.

Overlay assay

Protein samples from different conditions were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. Membrane was blocked with 5% non-fat milk in buffer PBST (PBS 0.05% tween 20). Hb (5 μg) 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. Finally, the antibody coupled to raddish peroxidase was added. The signal was developed by chemiluminescence ECL (Amersham) (Cruz-Castañeda and Olivares-Trejo 2008).

Results

An Hb-binding protein containing FRAP and NPNL motifs was identified in *H. pylori* genome

H. pylori is a pathogen that can support its growth using Hb as only iron source and express proteins capable of binding heme group, nevertheless, their identity remains unknown. To explore if *H. pylori* uses Hb as an iron source through of Hb-binding proteins we developed a strategy different that was reported by Worst et al. (1995). We performed an in silico analysis using as probe the amino acid sequence of ChuA (Expasy http://www.au.expasy.org/) with access number Q7DB97, which is an Hb-receptor reported in *E. coli* (Torres and Payne 1997). This strategy allowed us to identify in *H. pylori* proteome (Expasy http://www.au.expasy.org/) the FrpB2 protein with access number Q9ZKT4 and was annotated as a putative iron-regulated outer membrane protein. Multiple amino acid sequence alignment revealed the presence of FRAP and NPNL motifs necessary for Hb-binding. In addition, TonB motif was identified, it is crucial to obtain energy (Fig. 1).

FrpB2 is an *H. pylori* protein that conserves FRAP and NPNL motifs necessary for Hb-binding

To gain more information about the function of FrpB2 we compare FrpB2 and ChuA 3D structures by PyMol V 0.99 program. We observed that both 3D structures presented the same spatial conformation, the barrel structure typical of membrane proteins. The blue structure may function as a lid that allows the passing of iron source through of membrane. FRAP and NPNL motifs involved in Hb-binding are presented in yellow. This result suggests that FrpB2 protein presents a similar structure to ChuA and perhaps it has the same function as an Hb-binding protein (Fig. 2).

FrpB2 is an Hb-binding protein and is expressed in membrane

The fact that FrpB2 has the 3D structure like ChuA and it has FRAP and NPNL motifs, suggests that FrpB2 protein may act as an Hb-binding protein. To explore this assumption *frpB2* and *ChuA* genes were cloned under T7 promoter control and expressed in *E. coli* (BL21pLysS) no pathogenic. This bacterium lacks of genes that encode for Hb-binding proteins. Thereafter membrane and cytoplasmic fraction were separated by differential centrifugation. Thus, proteins were loaded onto SDS-PAGE and transferred to nitrocellulose membrane. The ability of binding Hb was tested by overlay assay as reported Cruz-Castañeda and Olivares-Trejo (2008). We did not

Fig. 1 FrpB2 protein contains FRAP/NPNL motifs necessary for Hb-binding. Multiple amino acid alignment. FrpB2 amino acid sequence of *H. pylori* genome was compared with sequences from ChuA (*E. coli*), HmuR (*Y. pestis*), HmuR (*P. gingivalis*), HmbR (*N. meningitidis*) to reveal the FRAP and NPNL motifs necessary for Hb-binding, they are shown in squares

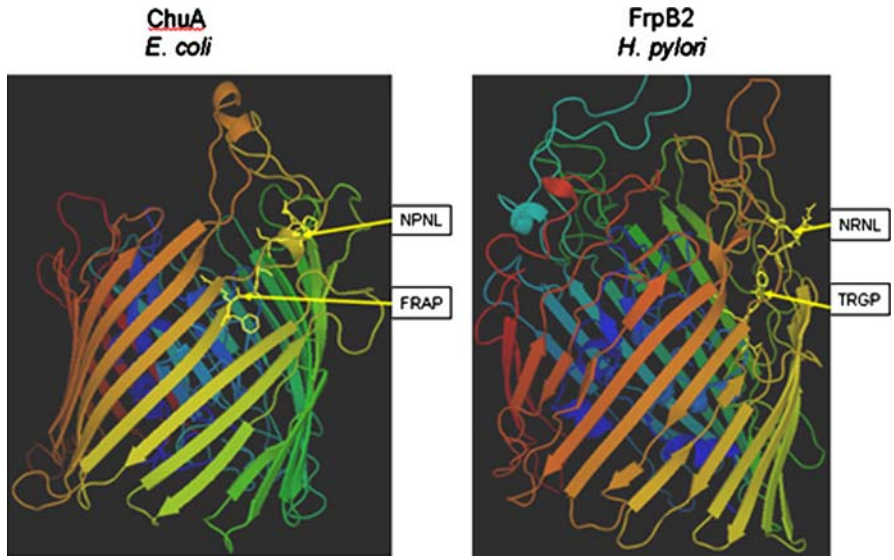

Fig. 2 ChuA and FrpB2 proteins have similar structure. ChuA and FrpB2 amino acid sequences were modeled using the PyMol program. Both proteins have the barrel structure. Inside the barrel a *blue* structure represents the lid, which is important to permit the passage through membrane of iron source. FRAP and NPNL domains are shown in *yellow*

detect Hb-binding proteins in the cytoplasmic fraction (Fig. 3a, lanes 2, 3). Substantially FrpB2 protein was detected in the membrane fraction and its molecular weight corresponded to estimate size (90.8 kDa) (Fig. 3b, lane 3) as well as the positive control (70 kDa) (ChuA) (Fig. 3b, lane 2). No detection was observed with negative control vector (Fig. 3b, lane 1) or with the negative control in which the overlay was performed without Hb (Fig. 3c). We performed overlay experiments using *H. pylori* membranes in order to explore whether this pathogen expresses Hb-bindig protein. Interestingly, a protein with an estimated size of 90.8 kDa was detected (Fig. 3d, lane 2), this protein was not observed in soluble fraction (Fig. 3d, lane 1) or when the overlay was performed without Hb (Fig. 3d, lane 3). These findings further suggest that FrpB2 protein is an Hb-binding protein and it is principally expressed in membrane.

The expression of *frpB2* and *ChuA* genes in *E. coli* supported the cell growth in media depleted of iron

The capability of FrpB2 to bind Hb suggests that it is involved in iron acquisition using Hb as an iron source. To explore more about this idea the function of *frpB2* gene was evaluated by growth kinetic. Cells transformed with different constructions were iron starved adding 2-2′dipyridyl as quelater, thereafter the media was supplemented with Hb or different metalloproteins and the cell growth was tested every 2 h by espectrophotometry. *E. coli* transformed with the empty vector did not grow in iron absence. Only cells transformed with pJET-ChuA (positive control) and pJET-frpB2 plasmids maintained the cell growth (Fig. 4a). These experiments support the idea that *frpB2* gene encodes a membrane Hb-binding protein involved in iron acquisition. To investigate whether FrpB2 utilizes other metalloproteins as an iron source we performed growth kinetic experiments as in A, but after iron starvation the medium was supplemented with metalloproteins different of Hb. Apo-transferrin, Holo-transferrin, Lactoferrin and Albumin were tested as only iron source and they did not support the cell growth (Fig. 4b, c). Both ChuA and FrpB2 proteins are too specific for human Hb because Hbs, like mouse or goat, did not support the cell growth either (Fig. 4b, c).

Discussion

In this work an Hb-binding protein involved in iron acquisition was identified by in silico analysis (FrpB2). This protein was annotated as a putative iron-regulated outer membrane protein and it presented the motifs necessary to bind Hb. The molecular weight estimated experimentally was 90.8 kDa, higher than reported for three proteins previously described (77, 50 and 48 kDa), which bind heme (Worst et al. 1995). Interestingly, we did not find those

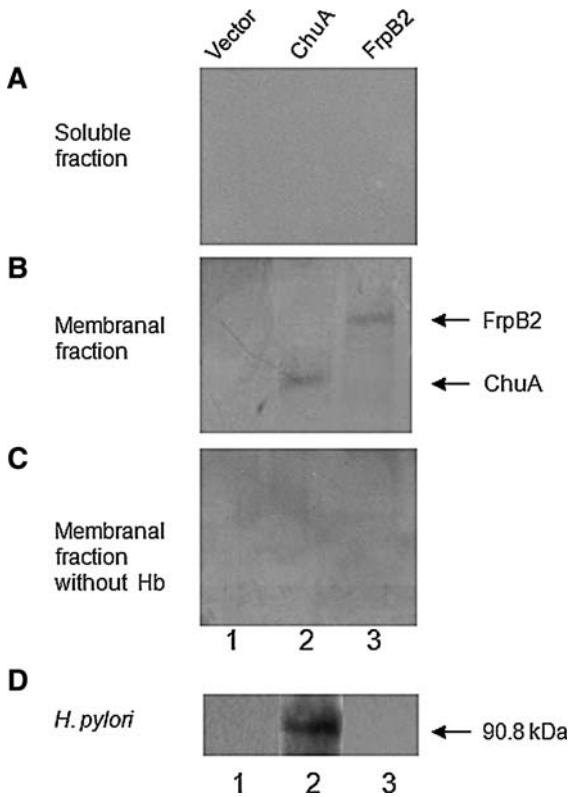

Fig. 3 *frpB2* gene encodes an Hb-binding protein expressed in membrane. pJETfrpB2, pJETChuA and pJET constructions transformed in *E. coli* were induced with 1 mM IPTG. Cells were collected and broken by French pressure. Cytoplasmic and membranal fractions were separated by ultracentrifugation. Samples of each fraction were load onto SDS-PAGE, transferred to nitrocellulose membrane and Hb-binding proteins were revealed by overlay assay using anti-β-globin antibodies. *Panel A*: cytoplasmic fraction (CF), *Panels B and C*: membranal fraction (MF), in C overlay assay was performed without Hb as a *negative control*. Lane 1: pJET, *negative control*, Lane 2: pJETChuA, Lane 3: pJETfrpB2, *positive control*. Estimated molecular weight for ChuA (70 kDa) and FrpB2 (90.8 kDa) is shown by the arrows. *Panel D*: overlay experiments were performed using *H. pylori* membranes. Lane 1: soluble fraction, Lanes 2 and 3: membranal fraction, but in lane 3, Hb was not added (*negative control*)

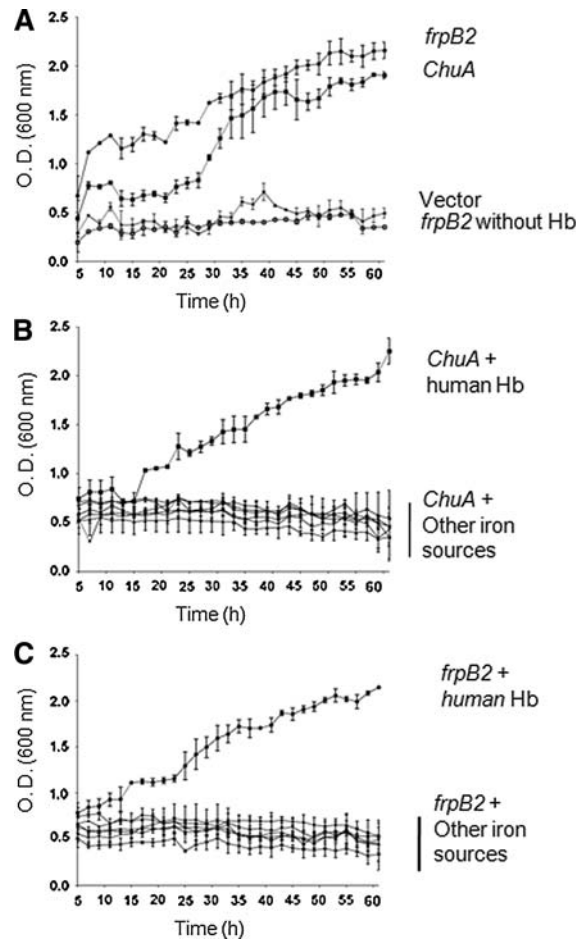

Fig. 4 The *frpB2* gene expression supports the *E. coli* cell growth using Hb as a sole iron source. BL21pLysS strain was transformed with pJET-frpB2, pJETChuA and pJET (vector) and cell growth was iron starved using 2-2′ dipyridyl and the media was supplemented with human Hb or different metalloproteins. Samples were taken every 2 h and the cell growth was tested by spectrophotometry. *Panel A*: pJETfrpB2 without Hb (O), pJETfrpB2 (●), pJETChuA (■), pJET (X). *Panel B*: pJETChuA after iron starvation, the medium was supplemented with different iron sources, (■) human Hb, (●) Apo-transferrin, (□) Holo-transferrin, (▲) Lactoferrin, (▼) Albumin, (◆) mouse Hb, (O) goat Hb, (Δ) without source (*negative control*). *Panel C*: as in B but the construction tested was pJETfrpB2

proteins with the strategy used in this study, maybe because those proteins are not related with the structure of FrpB2 protein and possibility they have a distinct role in the biology of *H. pylori*. We suggest that *frpB2* gene is involved in iron acquisition from human Hb specifically, because this gene was only able to maintain the *E. coli* cell growth in presence of human Hb. The identification of this protein in the membrane fraction suggested that FrpB2 could be an Hb-receptor protein like ChuA. This assumption was supported by 3D comparison, both proteins have similar structures and also FRAP/NPNL motifs are conformationally conserved. We think that FrpB2 could participate in a mechanism when *H. pylori* causes ulcers and perhaps this mechanism is distinct of that which is used by *H. pylori* when it is invading the gastric mucosa; here the principal iron source is lactoferrin protein. In summary, we propose that

FrpB2 is an Hb-binding protein expressed in membrane, it is involved in iron acquisition and it uses human Hb specifically.

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