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Helicobacter pylori secretes the chaperonin GroEL (HSP60), which binds iron

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ABSTRACT

***Helicobacter pylori* is a bacterium that can use multiple iron sources. However, it is unknown whether this bacterium secretes molecules such as siderophores or haemophores to scavenge iron. Here, we report the first secreted iron-binding protein of *H. pylori*, which we purified by haem-affinity chromatography. Mass spectrometry analysis revealed its identity as chaperonin (HpGroEL). When we compared HpGroEL with EcGroEL from *Escherichia coli*, they were homologous, showing 60% similarity. Additionally, purified cytoplasmic HpGroEL could also bind iron. Perhaps *H. pylori* secretes HpGroEL to maintain the appropriate folding of extracellular proteins and to bind iron.**

1. Introduction

Iron acquisition is potentially indispensable for nearly all organisms [1]. The efficiency of several metabolic processes, such as respiration and oxygen transport, depends on adequate iron levels [1]. Human pathogens often obtain necessary iron from human host sources such as lactoferrin (Lf) or transferrin (Tf), which include haemoglobin (Hb) or haem [2]. For pathogens to successfully obtain iron from host sources, they must develop multiple mechanisms to do so. One mechanism (the direct mechanism) consists of expressing membrane proteins (receptors) [3]; these proteins can directly bind the iron source. A different (indirect) mechanism involves secreting small non-protein molecules (siderophores) or proteins (haemophores) to scavenge iron and to deliver it to the bacterial membrane, where a protein receptor in the lipid bilayer binds and internalises the iron supply [4]. Despite significant amino acid sequence variation in these protein receptors among different pathogens, it has been proposed that some of these proteins bind the iron source via two motifs (FRAP and NPNL) [5,6]; one histidine (H) located between these motifs may interact with the iron molecule [7]. *Helicobacter pylori* is a pathogen frequently associated with gastric ulcers, and this bacterium has the special feature of being adapted to use Lf, Tf, haem and Hb as iron sources [8]. It is known that this bacterium expresses an Lf-binding membrane protein, giving *H. pylori* the ability to use Lf as an iron source [9]. Additionally, 3 haem-binding membrane proteins with sizes of approximately 48, 50 and 77 kDa have been linked to iron acquisition, but their identities remain unknown [10]. Two membrane proteins, termed FrpB1 (88.5 kDa) and FrpB2 (90.8 kDa), have also been identified. These proteins can bind Hb, and FrpB1 can also bind haem [11,12]. Finally, it is known that *H. pylori* can take up iron using a system of transporters termed FeoB [13]. Although there is abundant evidence that *H. pylori* can use iron, Lf, haem and Hb through a direct mechanism, by expressing at least eight membrane proteins that bind them, there is no evidence that *H. pylori* synthesises siderophores [10,14] and no reported secretion of haemophores to scavenge iron. In this work, we explore whether *H. pylori* can bind iron sources by an indirect mechanism that may be important for the bacterium and may participate during the infective process.

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2. Materials and methods

2.1. Bacterial growth conditions

H. pylori J99 (ATCC 700824) and *Escherichia coli O157:H7* (ATCC 700927) strains were used in this study. *H. pylori* strain J99 was routinely grown at 37 °C on blood agar plates (Casman agar containing 7.5% sheep blood) under microaerophilic conditions (10% CO_2) for 24 h at 37 °C before being grown on Casman agar plates containing 5 mM $FeCl_3$ for 12 h at 37 °C under microaerophilic conditions (10% CO_2).

E. coli O157:H7 strain *EDL 933* was grown on Luria–Bertani agar plates for 12 h at 37 °C, after which the bacteria were again grown on Casman agar plates containing 5 mM $FeCl_3$ for 12 h at 37 °C.

2.2. Isolation of secreted, total and cytoplasmic proteins

Both bacterial strains were grown on Casman agar plates supplemented with 5 mM $FeCl_3$, collected, suspended in Brucella Broth and cultivated for 12 h. Cells were then centrifuged at 6000×*g* for 10 min, and the supernatant was filtered through a 0.45-μm pore size membrane filter to remove residual bacteria from the supernatant containing secreted proteins. Cells were washed three times with Brucella Broth by centrifugation at 6000×*g* for 10 min and were finally resuspended in medium supplemented with 1 mM PMSF and 1% sarkosyl. Bacteria were lysed by sonication for 6 min with pulses of 30 s. To eliminate intact cells, samples were centrifuged at 12000×*g* for 20 min. The supernatant was considered to contain total proteins. To isolate cytoplasmic proteins, this supernatant was ultracentrifuged at 105000×*g* for 1 h at 4 °C, and the resulting supernatant was considered to contain cytoplasmic proteins [11].

2.3. Purification of proteins by affinity chromatography

Secreted, total or cytoplasmic proteins were loaded onto affinity chromatography resin coupled to haem (Sigma). The interaction was maintained overnight at 4 °C. The resin was centrifuged at 1000×*g* for 1 min, the flow-through was collected and the resin was washed four times with wash buffer at pH 7.0 (50 mM Tris–HCl, 100 mM NaCl) to eliminate unspecific interactions. Proteins bound to the haem were released with 6 M guanidine hydrochloride. The samples were cleaned of salts, lipids and nucleic acids by precipitation using a kit (Cleanup kit, Bio-Rad). Salts and other contaminants remained in the supernatant, and the proteins were recovered in the pellet.

2.4. Protein quantification and Coomassie brilliant blue staining

The proteins resolved on gels and used for subsequent mass spectrometry analysis were visualised by a Coomassie brilliant blue R-250 method; the gel was immersed in a staining solution (0.1% Coomassie brilliant blue R-250, 50% methanol and 10% glacial acetic acid) for 15 min with gentle agitation and then destained in a destaining solution (40% methanol and 10% glacial acetic acid) until the gel background was fully clear. When the silver staining method was used, the gel was immersed in a fixing solution (40% ethanol, 5% glacial acetic acid) overnight, and then a fixing step was performed. The gel was washed twice (20 min each) with 50% ethanol and three times with deionised water for 5 min. The gel was then pre-treated (0.02% $Na_2S_2{\cdot}5H_2O$ for 2 min) and washed three times in deionised water for 20 s. The gel was stained with a staining solution (0.2% $AgNO_3$ and 40 μL of 37% formaldehyde) for 30 min and washed two times (distilled water) for 20 s. The result was revealed by incubating the gel in developing solution (3% Na_2CO_3, 0.0004% $Na_2S_2{\cdot}5H_2O$ and 40 μL of 37% formaldehyde). The gel was gently agitated until the bands appeared, at which point the reaction was stopped by adding 5% glacial acetic acid and incubating for 10 min. The concentration of proteins was determined by spectrophotometry using the Bradford method; a standard curve was generated using bovine serum albumin. The concentration of proteins, read at 595 nm in a spectrophotometer, was determined by interpolation. For Coomassie blue-stained 10% SDS–PAGE, 50 μg of protein was loaded onto gels, while for silver staining, 5 μg of protein was loaded.

2.5. Western blotting

No bacterial lysis occurred when bacteria were cultivated for 12 h. *H. pylori and E. coli* were cultivated for only 12 h to prevent cell lysis [15]. A Western blot was performed using anti-β-galactosidase antibodies as an internal control to verify that no intracellular proteins were released under our culture conditions. Proteins were transferred to nitrocellulose membranes in a semi-dry trans-blot cell for 1 h at 100 mA in a solution of 30 mM Tris and 20% methanol. The membranes were soaked in PBST buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 and 0.1% Tween 20) containing 5% non-fat milk overnight to saturate all remaining active binding sites. The membranes were washed three times with PBST and then incubated with anti-GroEL antibody (Enzo Life Sciences) (1:40000) or with anti-β-galactosidase antibody (Millipore) (1:50000) for 1 h. Five washes were performed with PBST, and the secondary antibody was added (anti-rabbit coupled to horseradish peroxidase; 1:10000) [12]. Both antibodies were specific for *E. coli* proteins but cross-react with homologous proteins from *H. pylori* [16].

2.6. Competition experiments

Competition experiments were performed identically to affinity chromatography purification, but the proteins bound to the haem-affinity resin were released with haem, $FeCl_3{\cdot}6H_2O$, $ZnCl_2$, $MnCl_2{\cdot}4H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$, vitamin B_{12} and Lf. The eluents (except guanidine hydrochloride) had a concentration that was twofold greater than the iron concentration of the haem-affinity resin. Each sample (5 μg) was subjected to SDS-PAGE.

2.7. Mass spectrometry analysis

After destaining the Coomassie blue-stained SDS-PAGE gel, the band corresponding to 58 kDa was cut, washed with deionised water and enzymatically digested with trypsin. LC–MS/MS was performed on a Micromass QTof I equipped with an LC Packings nanoflow LC. The digested solution (5 μL) was injected onto an LC Packings C18 PepMap column (0.75 μm × 15 cm) and eluted with a linear acetonitrile gradient at a flow rate of 200 nL/min. The peptides eluting from the column were introduced into the mass spectrometer through a new objective PicoTip held by a new objective adapter. The experimental conditions were as follows: capillary voltage 1.8 kV, cone voltage 32 V and collision energy from 14 to 50 eV according to the mass and charge of the ion. Raw data files were processed using the MassLynx ProteinLynx software, and pkl files were submitted for searching at www.matrixscience.com using the Mascot algorithm (Protein Core Facility, Columbia University Medical Center, http://www.cumc.columbia.edu/dept/protein/).

2.8. Amino acid sequence alignment

The amino acid sequence of the HpGroEL protein (from *H. pylori J99*) was compared with GroEL protein sequences from other bacteria (*Helicobacter pylori 26695*, *Helicobacter pylori HPGA1*,