Multiple haem-utilization loci in *Helicobacter pylori*

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To identify genes responsible for the utilization of haem as an iron source in *Helicobacter pylori*, a siderophore synthesis mutant of *Escherichia coli* was transformed with an ordered cosmid library of *H. pylori* NCTC 11638. Four independent cosmids were found that were able to complement this mutant on iron-restrictive solid media containing different haem compounds as the sole source of iron. Hybridization experiments revealed that the four cosmids contained unrelated DNA fragments. No major differences were observed in the growth of the four transformants on iron-restrictive solid media to which different haem compounds had been added. None of the cosmids could confer the ability to use haem as an iron source to an *E. coli* aroB tonB mutant, which means that transport of iron and/or haem across the outer membrane requires a functional TonB protein. Further characterization of the cosmids revealed that one of them was also able to complement *E. coli* aroB hemA, indicating that the haem molecule is taken up as a whole by this haem-biosynthesis mutant. Expression of this haem-uptake system could not be repressed by excess iron. Another cosmid expressed two polypeptides in *E. coli* which were specifically immunoreactive with a polyclonal antiserum raised against whole cells of *H. pylori*. The production of these proteins appeared to be iron repressible. One of these proteins has the same molecular mass as a previously described 77 kDa haem-binding iron-repressible outer-membrane protein (IROMP) of *H. pylori*.

Keywords: *Helicobacter pylori*, multiple haem-utilization loci, iron regulation, TonB-dependent transport

INTRODUCTION

*Helicobacter pylori* is a spiral-shaped, Gram-negative bacterium found predominantly in the mucus layer of the human stomach (Lee *et al.*, 1993). Infection is strongly associated with type B antral gastritis and peptic ulcer disease, and may be the initiation factor of gastric cancer (Blaser, 1990). The bacteria are highly motile and their spiral shape enables them to migrate easily, deep into the viscous mucus layer. Here, the organisms tend to congregate at a specific site, the intercellular junctions of the epithelial cells (Hazel *et al.*, 1986). The mucus layer provides the micro-organism with a microaerophilic environment and protection from the acidic environment of the stomach by creating a pH gradient leading to a neutral pH at the epithelium surface (Fauchere *et al.*, 1989).

However, this neutral pH environment comes at the cost of being an iron-deficient niche, as a result of the iron-withholding defence system of the human mucosa (Weinberg, 1984; Otto *et al.*, 1992). Bacteria have developed a variety of mechanisms to circumvent this defence system of the host: directly by binding transferrin or lactoferrin, indirectly by a siderophore-mediated process, or by binding of haem and/or haem–protein complexes (Mietzner & Morse, 1994). The largest source of iron in mammals is found intracellularly as haemoglobin; red blood cells contain approximately 60% of all the available iron in the human body (Welch, 1992). Any free haem or free haemoglobin is bound by protein carriers: haptoglobin,
haemopexin, and serum albumin (Muller-Eberhard & Morgan, 1975). However, upon lysis of an erythrocyte, the concentration of haptoglobin (12 µM) may be much too low to bind the released intraerythrocytic hae-moglobin, which can approach 20 mM (Welch, 1992). Because of this abundance of haem-iron compounds in the host, it is not surprising that many bacterial pathogens have developed specific mechanisms for utilization of haemoglobin or other host haemoproteins as an iron source. In *Vibrio cholerae*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Haemophilus ducreyi*, *Shigella dysenteriae* and *Yersinia enterocolitica*, specific receptors are involved in binding haemoglobin, haemoglobin or haemoglobin–haem complexes (Stojiljkovic & Hanrke, 1992; Henderson & Payne, 1993, 1994; Wong et al., 1994; Cope et al., 1995; Elkins, 1995; Mills & Payne, 1995; Stojiljkovic et al., 1995). In most cases, these receptors mediate uptake of haem in a TonB-dependent manner. The entire haem molecule can be transported into the cell or, alternatively, only the iron is transported into the cell and not the protoporphyrin portion of the haem molecule (Desai et al., 1995).

The possession of a haem-uptake system has significant advantages, especially for bacteria growing on mucosal surfaces. Firstly, haem compounds are present due to the desquamation of the epithelial cells. Secondly, the haem-scavenging systems of the host mucosal cells are not very efficient. Furthermore, for *H. pylori*, which congregates at the intercellular junctions of the epithelial cells, haem compounds from epithelial cells and the bloodstream could be easily accessible because of leakage through the junctions. The production of haemolysin and cytoxin (Leunk, 1991; Segal & Tompkins, 1994) could further contribute to this leakage of haem compounds. It has been shown that haemolin can serve as the sole iron source for *H. pylori* and several iron-regulated haemin-binding proteins have been found (Worst et al., 1995). These facts, together with the absolute need for haem as a growth factor for *H. pylori* even in the presence of abundant iron (Hassel et al., 1986), may suggest the existence of a specific haem-(iron)-uptake system in *H. pylori*.

In this study, *Escherichia coli* EB53 (*aroB hemA*), a K-12 derived strain in which the *aroB* and *hemA* mutations disrupt synthesis of the siderophore enterobactin and haem respectively (Eberspächer & Braun, 1980), was used for the identification of *H. pylori* haem-(iron)-uptake systems. Strain EB53 does not grow on iron-restricted media and is unable to transport haemoglobin across its outer membrane. Complementation of EB53 with an ordered cosmid library from *H. pylori* NCTC 11638 led to the identification of four distinct genomic loci that are responsible for haem utilization in *H. pylori*.

**METHODS**

**Bacterial strains, media and culture conditions.** *E. coli* strain DH5α, which contains the ordered cosmid library of *H. pylori* NCTC 11638 (Bukanov & Berg, 1994), was grown in Luria-Bertani (LB) broth containing 25 µg kanamycin ml−1. *E. coli* strains EB53 *aroB hemA* (Eberspächer & Braun, 1980) and IR5740 *aroB hemA tonB*::Kan (Stojiljkovic et al., 1995) were grown in LB supplemented with 50 µg 5-aminolaevulinic acid ml−1 (ALA; Sigma). *H. pylori* cells were grown on Columbia agar containing 5 % lysed horse blood and Dent supplement (Oxoid) under microaerophilic conditions. Iron-restricted growth of *H. pylori* was performed in brucella broth with 20% newborn calf serum (NBCS; B520) as described previously (Worst et al., 1995). Iron-replete conditions were obtained by the addition of iron(III) nitrate to B520 in a final concentration of 1 mM. The iron-restricted medium used for analysis of utilization of different haem compounds by EB53 harbouring various cosmids was nutrient broth (Oxoid) with 0.3 mM dipyridyl (BDH Chemicals), solidified with 1.5 % Bacto-agar (NBD plates; Stojiljkovic & Hanrke, 1992).

**Utilization of haem compounds.** Haemoglobin (Sigma) was dissolved in 0.1 M NaOH, filter-sterilized and a final concentration of 30 µg ml−1 (~ 50 µM) was used in the NBD plates for the complementation experiments. Horse haemoglobin (Serva) was dissolved in water, filter-sterilized and used in a final concentration of 500 µg ml−1 (~7 µM). These concentrations were chosen because they were used by other authors in a similar approach (Stojiljkovic & Hanrke, 1992; Elkins, 1995). Horse blood was obtained from Biocoradning, Mijdrecht, the Netherlands. Horse blood was lysed by addition of 0.2% (w/v) saponin (Merck) and incubation for 30 min at 37 °C. Horse blood and lysed horse blood were used in final concentrations of 5% (v/v). NBCS (Gibco) was used in a final concentration of 10% (v/v). These quantities of different haem compounds were chosen because they give good growth of *H. pylori* on these media.

For unknown reasons we were unable to get reproducible data when we measured optical densities from liquid cultures of EB53 transformants. Therefore growth rates could not be assessed as increased optical densities of liquid cultures, but instead were determined as growth on solidified medium. For this, 100 µl of iron-starved (liquid NBD with ALA and kanamycin) overnight cultures of EB53 transformants were plated on NBD agar containing the different haem compounds. Growth was scored daily for up to 4 d.

**Recombinant DNA techniques.** We used the ordered cosmid library of Bukanov and Berg in our complementation experiments. This library contains 68 cosmids with Sau3AI partial-digested DNA fragments of approximately 40 kb cloned in the low copy vector Lorist6 (Bukanov & Berg, 1994). The cosmids were extracted and purified with plasmid spin columns as described by the manufacturer (QIagen). *E. coli* EB53 and IR5740 were transformed using a standard electroporation protocol for *E. coli* (Maniatis et al., 1982). For Southern blot hybridization analysis, restriction-enzyme-digested cosmids were electrophoresed through a 0.8% agarose gel, transferred to a nylon filter (Boehringer Mannheim) by capillary blotting and cross-linked to the filter by UV irradiation for 3 min.

For RNA spot-blot analysis, total RNA was isolated from iron-restricted and iron-replete *H. pylori* cells (approx. 106 cells), using the RNEasy kit of Qiagen. The RNA was treated with RNase-free DNase (0.1 unit per µg RNA; Stratagene). We estimated the amounts of RNA after agarose gel electrophoresis and staining with ethidium bromide. Amounts of 100, 10, 1 and 0.1 ng in a total volume of 50 µl 20 × SSPE (1 × SSPE is 180 mM NaCl, 10 mM sodium phosphate pH 7.5, 1 mM EDTA) were spotted on nylon filters (Boehringer Mannheim) using a spot-blot apparatus (Bio-Rad). To verify
that equal amounts of RNA had been spotted from iron-restricted and iron-replete cells, a 850 bp PCR fragment, derived from the 23S rRNA gene of \(H\). \(p\)ylori, was used as a probe. DNA probes were labelled with \([\alpha^32\text{P}]\)dATP (Amersham) using a random-prime labelling kit (Prime-it II; Stratagene). Hybridization of blots was performed overnight at 68 °C in hybridization solution: 1 % (w/v) sodium dodecyl sulfate (SDS), 0.1 % (w/v) sodium lauryl sarcosinate (Sarkosyl; Ciba-Geigy), 1 % (w/v) blocking reagent (Boehringer Mannheim) and 6 x SSPE. Washings were performed at room temperature (2 x 10 min) with 2 x SSPE, 1 % (w/v) SDS and 68 °C (2 x 10 min) with 1 x SSPE, 1 % (w/v) SDS. Spot hybridization was quantified with a phosphorimager (Molecular Dynamics). Radioactivity counts of the cosmids hybridizing with the spots were determined by scanning the blots with Imagequant (Molecular Dynamics). By integration of the areas of the count peaks, total hybridization counts of the RNA spots were determined, from which a hybridization ratio could be calculated.

SDS-PAGE and immunoblot analysis. Outer-membrane proteins from iron-restricted and iron-replete \(H\). \(p\)ylori cells were isolated using Sarkosyl as previously described (Worst et al., 1995). EB53 transformants were cultured overnight in 10 ml iron-restrictive liquid NBD, supplemented with ALA and 25 µg kanamycin ml \(^{-1}\). Total proteins were extracted by solubilizing the pelleted bacteria in 100 µl SDS sample buffer (0.0625 M Tris/HCl pH 6.8; 2 %, w/v, SDS; 10 %, v/v, glycerol; 0.001 %, w/v, bromophenol blue). Outer-membrane proteins from \(H\). \(p\)ylori and total proteins from EB53 were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Schleicher and Schuell) as described previously (Worst et al., 1995, 1996). Blocking of non-specific binding sites was performed with 0.5 % (w/v) blocking reagent (Boehringer Mannheim) in Tris-buffered saline (TBS: 20 mM Tris, 500 mM NaCl, pH 7.5) for 45 min. The blot was incubated overnight with a rabbit polyclonal antiserum against formalin-killed whole cells of \(H\). \(p\)ylori ATCC 43504, diluted 1:100 in TBS with 0.25 % (w/v) blocking reagent under constant rocking at 37 °C. The membrane was washed with TBS containing 0.05 % Tween (TBST, 3 x 15 min) and incubated for 1 h with horseradish-peroxidase-conjugated goat anti-rabbit IgG (Nordic Immunology) in TBST with 0.5 % normal goat serum at a dilution of 1:1000. After washing with TBST, colour was developed with 25 ml citrate/phosphate buffer containing 15 mg 4-chloro-1-naphthol (Bio-Rad) and 12.5 µl 30 % \(H_2\text{O}_2\); staining was stopped by rinsing with water.

RESULTS

Complementation of EB53 with an ordered cosmid library of \(H\). \(p\)ylori NCTC 11638

To identify haem-iron-uptake system(s) in \(H\). \(p\)ylori, we used an approach previously described to identify haemin/haemoglobin receptors in \(Y\). \(e\)nterocolitica and \(V\). \(c\)holerae (Stojiljkovic & Hancke, 1992; Henderson & Payne, 1994) and a haemin-uptake locus in \(Y\). \(e\)ntermisia \(p\)estis (Hornung et al., 1996). The \(n\)emA mutation of EB53 disrupts synthesis of haem and the \(a\)roB mutation prevents synthesis of the siderophore enterobactin. For its growth, EB53 requires both the haem biosynthetic precursor ALA and a non-haem iron source (Eber-spächer & Braun, 1980). Since we were primarily interested in utilization of haem as an iron source, ALA was added to the iron-restrictive media. If provided with gene(s) encoding the use of haem or haem compounds as an iron source, EB53 with the \(a\)roB mutation will be able to grow on iron-restrictive media only with exogenously supplied haem compounds. The 68 cosmids from the ordered cosmid library of \(H\). \(p\)ylori NCTC 11638 were isolated and combined into five pools: pool 1 (1–14), pool 2 (15–28), pool 3 (29–42), pool 4 (43–56) and pool 5 (57–68). EB53 was electrotransformed with each DNA pool and plated on NBD plates with ALA and kanamycin. After 3 d, colonies could be detected on plates with cells electroporated with pool 1, pool 2 and pool 5. No colonies were detected on plates with cells electroporated with pool 3 or pool 4. As a control, EB53 electroporated with sterile water was plated on the same plates without kanamycin. There was no growth of EB53 on these plates. Subsequently, all cosmids from pools 1, 2 and 5 were tested individually for their ability to complement EB53 for haem-iron uptake. Pool 1 contained two complementing cosmids (2 and 13) and pool 2 and 5 one each (16 and 61 respectively).

Growth of transformants with different iron- and haem-containing compounds

To obtain some information on the relative growth of the four different EB53 transformants (2, 13, 16, 61) on iron-restricted media containing different haem compounds, we plated them in equal amounts on NBD plates containing ALA, kanamycin and one of the following compounds: haemin, horse haemoglobin, horse blood, lysed horse blood and NBCS. No growth of untransformed EB53 was detected on these haem-containing media, indicating that the concentration of dipyridyl in the plates is sufficient to chelate possible inorganic iron contamination from the stock solutions used.

After 1 d incubation, pinpoint colonies (approx. 0.2 mm) of the EB53 transformants were detected on the agar plates that contained whole blood and serum, and slightly bigger colonies on plates with lysed blood. On haemoglobin and haem media, pinpoint colonies were only detected after 2 and 3 d, respectively.

Haem specificity and dependency of EB53-complementing transformants

Because we transformed almost completely the entire genome of \(H\). \(p\)ylori into EB53, it could be expected that one of the complementing cosmids contained \(H\). \(p\)ylori \(a\)roB. To check for this, the transformants were first plated on NBD plates with ALA and kanamycin but without an additional haem-iron compound. It is important to note that, whilst certain \(H\). \(p\)ylori genes may be well expressed in \(E\). \(c\)oli EB53, other genes may not be expressed, e.g. due to differences in promoter structure. Only if an EB53 transformant contains and expresses the \(H\). \(p\)ylori \(a\)roB would it grow on the above-mentioned plates. It appeared that only transformant EB53-16 could grow on this medium (Table 1).
Table 1. Growth of EB53 transformants on solid media

<table>
<thead>
<tr>
<th>Medium</th>
<th>-Fe + ALA</th>
<th>-Fe - ALA</th>
<th>+Fe - ALA</th>
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<tbody>
<tr>
<td>EB53 transformants</td>
<td></td>
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</tr>
<tr>
<td>LB</td>
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<td>NB</td>
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<tr>
<td>NB haemin</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>NB haemoglobin</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>NB lyzed blood</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>NB whole blood</td>
<td>+</td>
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</table>

+ , Visible colonies on the plates detected within 4 d of incubation; −, no visible colonies after 4 d incubation. +AL and −AL, addition and no addition of 5-aminolaevulinic acid respectively. −Fe, iron restriction by addition of 0.3 mM 2,2-dipyridyl; +Fe, no addition of 2,2-dipyridyl.

Table 2. Growth of transformed and untransformed E. coli mutants on NBD plates

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>NBD plates supplemented with</th>
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<tr>
<td></td>
<td>Haemin</td>
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<tr>
<td>EB53 (aroB hemA)</td>
<td>−</td>
</tr>
<tr>
<td>EB53 transformants*</td>
<td>+</td>
</tr>
<tr>
<td>IR5740 (aroB hemA tonB)</td>
<td>−</td>
</tr>
<tr>
<td>IR5740 transformants*</td>
<td>−</td>
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</table>

*EB53 and IR5740 transformed with cosmids 2, 13, 16 or 61. All transformants showed the same phenotype.

Subsequently the transformants were also plated on NBD plates with haem compounds but without ALA. If EB53-16 contained H. pylori aroB it would never be able to survive on plates without ALA, because H. pylori aroB cannot complement the hemA mutation in EB53. It appeared that EB53-16 could grow without ALA (Table 1), but only on blood-containing media and not with haemin or haemoglobin as the only haem compound added. To determine whether utilization of haem on ALA-deficient, blood-containing media by this clone is iron regulated, an excess of iron(III) nitrate was added. This iron replenishment did not influence growth of EB53-16 on these media.

**TonB dependency of haem-iron utilization by EB53 transformants**

In many iron-uptake systems in different bacteria, the TonB protein has been found to be essential for iron and/or haem transport across the outer membrane (Hussein et al., 1981; Braun et al., 1991; Segal & Tompkins, 1994). To determine whether utilization of haem-iron by our four transformants was dependent on a functional TonB protein, a tonB-negative mutant of EB53 (IR5740) was transformed with the four complementing cosmids. EB53 and IR5740 transformants, together with the untransformed strains, were tested on NBD plates containing ALA and different iron/haem compounds. Due to the aroB mutation, neither EB53 nor IR5740 is able to grow on iron-restrictive media containing haemin or haemoglobin as the only iron source. When transformed with H. pylori cosmids 2, 13, 16 or 61, EB53 was able to grow on these media but IR5740 was not. In contrast, all strains did grow in the presence of excess inorganic iron (iron(III) nitrate), which is not taken up in a TonB-dependent manner but probably by diffusion through the porin channels. To confirm the TonB phenotype of IR5740, growth was tested on media containing iron(III) citrate, since uptake of iron(III) citrate is a TonB-dependent process in E. coli (Hussein et al., 1981). IR5740 and IR5740 transformants did not grow on these media, whereas EB53 and EB53 transformants did (Table 2).
Cross-hybridization of complementing cosmids

To assess whether utilization of haem-iron by *H. pylori* was conferred by different, unrelated DNA segments, each of the four complementing cosmids was used as a probe against a Southern blot that contained the DNA from these four cosmids. We found that each cosmid only hybridized with its own insert DNA, suggesting that the cosmids contain different genes for haem utilization (not shown).

Iron regulation of transcription from complementing cosmids

To determine whether the haem-utilization genes on the four different complementing cosmids are iron regulated, radiolabelled cosmids were hybridized with equal amounts of total RNA from *H. pylori* cells cultured under iron restriction and iron repletion. The hybridization ratio between cosmids hybridizing with RNA from iron-restricted and iron-replete cells was calculated as an indication of iron repression. As shown in Fig. 1, the data indicate that for all cosmids there was a stronger hybridization signal with the iron-restricted RNA spots than with the RNA spots from iron-replete cells. However, large differences were found in the hybridization ratios of the different cosmids, which were calculated by integration of the peak areas from Fig. 1. Cosmid 61 showed the highest ratios (2.5 ± 0.4). The hybridization ratios of cosmid 16 were lowest; 1.1 (±0.2). Hybridization ratios of cosmids 2 and 13 were 1.8 (±0.1) and 1.5 (±0.1) respectively.

Immunoreactivity of EB53 and transformant proteins with a polyclonal antiserum against whole cells of *H. pylori*

Total proteins of EB53 and the four haem-utilization transformants cultured in NBD medium with haemin were run together with outer-membrane preparations of iron-restricted and iron-replete *H. pylori* cells on a SDS-PAGE gel, blotted and incubated with rabbit *H. pylori* antiserum. The outer-membrane proteins derived from iron-restricted *H. pylori* cells showed some additional immunoreactive bands, which have been characterized previously as iron-repressible outer-membrane proteins.
against formalin-killed \textit{H. pylori} cells with: outer-membrane proteins from iron-restricted \textit{H. pylori} cells (lane 1) and iron-replete \textit{H. pylori} cells (lane 2); total proteins from iron-restricted EBS3-61 (lane 3) and EBS3 (lane 4); total proteins of iron-replete EBS3-61 (lane 5) and iron-restricted EBS3-61 (lane 6). Proteins of \textit{H. pylori} expressed in EBS3-61 are indicated with arrows. Molecular size markers are shown on the right.

Fig. 2. Immunoreactivity of a rabbit polyclonal antiserum raised against formalin-killed \textit{H. pylori} cells with: outer-membrane proteins from iron-restricted \textit{H. pylori} cells (lane 1) and iron-replete \textit{H. pylori} cells (lane 2); total proteins from iron-restricted EBS3-61 (lane 3) and EBS3 (lane 4); total proteins of iron-replete EBS3-61 (lane 5) and iron-restricted EBS3-61 (lane 6). Proteins of \textit{H. pylori} expressed in EBS3-61 are indicated with arrows. Molecular size markers are shown on the right.

(IROMPs) (Worst et al., 1995, 1996; Fig. 2, lane 1). One of these IROMPs, with a molecular mass of 77 kDa, was previously shown to bind haem protein. Furthermore, we showed that this protein is expressed in vivo and contains conserved immunogenic domains (Worst et al., 1996).

In EBS3 transformed with cosmid 61, an additional reactive band of approximately 50 kDa was detected with the \textit{H. pylori} antiserum (Fig. 2, lane 3), which was not present in cell lysates of untransformed EBS3 (Fig. 2, lane 4). We could not detect any additional bands in cell lysates from EBS3 transformed with cosmids 2, 13 and 61 (data not shown).

To see whether the expression of this \textit{H. pylori} protein in EBS3 is iron regulated, EBS3-61 was cultured in NBD (iron restricted) and in NB (iron replete) and the cell lysates were run next to each other (Fig. 2, lanes 5 and 6 respectively). Again, the additional 50 kDa protein (but also a faint additional band of 77 kDa) was detected in the iron-restricted cell lysates in lane 6. This protein has the same molecular mass as the 77 kDa IROMP of \textit{H. pylori} (Fig. 2, lane 1). We could not clearly determine whether the 50 kDa protein expressed in EBS3-61 co-migrates with an IROMP of \textit{H. pylori}.

\section*{DISCUSSION}

From an ordered cosmid library of \textit{H. pylori} NCTC 11638 four DNA fragments were isolated that complemented the inability of the \textit{E. coli} siderophore synthesis mutant EB53 to grow on iron-restricted media supplemented with either haemoglobin or other haem compounds. The growth kinetics of the transformant colonies indicate that serum and blood are more efficient haem-iron sources than haemoglobin and haemin. Probably the presence of several haem-protein carriers in blood and serum keeps haem and haemoglobin in a soluble and stable form and renders them more accessible to the bacterium. This observation is not unique for \textit{H. pylori}; that haemoglobin is a better iron source for bacteria than haemoglobin has also been reported for other bacteria (Stojiljkovic et al., 1995; Hornung et al., 1996).

The DNA fragments were mapped on four different, unrelated cosmids of \textit{H. pylori} NCTC 11638 (cosmids 2, 13, 16 and 61). The cosmids could not complement an \textit{E. coli aroB tonB} mutant, which suggests that in \textit{E. coli} utilization of haem as an iron source is dependent on a functional TonB protein. Moreover, it also indicates that complementation of EBS3 requires an active transport process and is not the result of leakage of haem into these cells.

EB53-16 is the only transformant that grew on NB and LB without any additional haem compound. This might be explained by the production of enterobactin due to the complementation of the \textit{aroB} mutation by \textit{H. pylori aroB}. However, this is unlikely because this transformant could also grow on haem-containing media without the addition of ALA. This indicates that (i) the \textit{hemA} mutation of EBS3 is complemented by a transport system from \textit{H. pylori} that transports the entire haem molecule into the cell; and (ii) \textit{H. pylori aroB} is either not present in the cosmid library, or is not expressed in \textit{E. coli}. Traces of haem in LB and NB could be sufficient to provide for the iron need of EB53-16 but not for its porphyrin need (Table 1). Only the highest haem concentrations, present in blood and lysed blood, are enough to satisfy its porphyrin need (Table 1). Haem uptake by this transformant on ALA-deficient media does not seem to be iron regulated because growth on the blood-containing media was not affected by the concentration of free iron.

Additional indications that the haem-uptake system on cosmid 16 is not affected by the environmental iron concentration was provided by RNA spot-blot hybridization experiments (Fig. 1). Cosmid 16 hybridized hardly better with RNA from iron-restricted cells. However, this observed lack of iron regulation is inconclusive since unregulated mRNAs could mask expression of an iron-regulated operon in the ~40 kb region. A potential reason for \textit{H. pylori} to constitutively express this haem-utilization system, even when sufficient iron is present, is that haem is an essential growth factor of \textit{H. pylori}. Without additional haem compounds (blood, serum), \textit{H. pylori} cannot grow on solid media (Hazell et al., 1986). Since haem cannot leak into bacteria through its porins, this haem-uptake system must be expressed constitutively, thus providing for the porphyrin need of the cell under any conditions. Obviously, utilization of haem by this haem-porphyrin-
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uptake system could also be very useful under iron-restricted conditions.

Cosmid 61 hybridized almost three times more with RNA from iron-restricted *H. pylori* cells than with RNA from iron-replete cells, indicating that *H. pylori* transcribes three times more mRNAs from genes present on cosmid 61 when cultured under iron restriction (Fig. 1). However, since the observed increase of mRNA transcription is the combined effect of all mRNAs that are being transcribed from this cosmid, our finding does not prove that the mRNA that encodes the complementing protein(s) is transcribed at a higher level during growth under iron-limited conditions. To determine the effect of iron limitation on the transcription of the complementing gene we would need to obtain the smallest subclone that confers the ability to grow under iron-restricted conditions. We tried to do this but did not succeed in obtaining stable subclones, due to the necessity to perform a functional assay (growth under iron-restricted conditions) in *E. coli* EB53, which is a RecA+ strain. These strains are known to display frequent rearrangements of cloned DNA.

Additional evidence that iron-induced proteins are being encoded by cosmid 61 comes from our Western blot data (Fig. 2). Two additional polypeptides expressed only in iron-restricted EB53-61 were recognized by a polyclonal antiserum against whole cells of *H. pylori*. Expression of these 50 kDa and 77 kDa proteins was iron regulated in *E. coli*. Furthermore, the 77 kDa protein has the same molecular mass as a previously described (Worst et al., 1995) IROMP of *H. pylori*, which is capable of binding haem. The 50 kDa protein expressed in EB53-61 could be the same protein as another previously described haem-binding IROMP of *H. pylori* which also has the same molecular mass.

No additional reactive band could be detected in EB53-2, 13 and 16 using the polyclonal antiserum against whole cells of *H. pylori* ATCC 43504. Several reasons can be suggested for this. First, expression of *H. pylori* proteins in *E. coli* may be too low for detection on immunoblot. Second, the serum was raised against whole cells of *H. pylori*, so probably only antibodies against outer-membrane components are present in the serum. Third, some haem-utilization proteins may not be immunogenic. Finally, it is possible that our antiserum, which was originally raised against *H. pylori* ATCC 43504, does not cross-react with the *H. pylori* NCTC 11638 proteins expressed in the EB53 transformants.

We conclude that *H. pylori* has at least four haem-iron-utilization systems in which several haem compounds can be used as the sole iron source. We postulate that one haem-uptake system is expressed constitutively for the uptake of the intact haem molecule, which is primarily essential for the cell’s porphyrin need. We also propose that at least one other haem-iron-utilization system contains iron-regulated genes and can be used by *H. pylori* under iron-restricted conditions. However, as discussed above, our indication for the iron-regulated character of these systems comes from RNA hybridization data using the complete cosmid as a probe, and hence it is not conclusive.

Since the cosmids containing the genes of these three iron-regulated systems were unable to complement the hemA defect of EB53, it is unlikely that these systems import an intact haem molecule into the cytoplasm. More likely the haem-iron is already removed from the porphyrin ring at the cell surface or prior to entry into the cytoplasm. This phenomenon has already been described for haemin uptake by *Neisseria gonorrhoeae* (Desai et al., 1995). Another possibility is that the haem molecule is taken up intact but subsequently degraded during its transport into the cytoplasm by a haem-oxygenase system.

The possession of multiple (haem)-iron-acquisition systems by a bacterium like *H. pylori* makes sense because *in vivo* it encounters different environments that vary not only in iron and haem content but also in iron and haem composition. These different haem compounds might be used as an iron source through different acquisition systems. Furthermore, based on sequence homologies, the genome of *H. pylori* contains several distinct loci that contain genes that share homology with TonB-dependent haem/iron-uptake systems described in other bacteria (Tomb et al., 1997). Five of them are homologues of FrpB, a protein that may help to obtain iron from haem and lactoferrin (Berg et al., 1997; Tomb et al., 1997).

The possession of different haem-utilization systems could greatly contribute to the virulence of *H. pylori* since the ability of pathogenic micro-organisms to scavenge iron and/or haem compounds from their host environment is fundamental for survival in the host and thus production of disease.

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**REFERENCES**


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