The Role of the Ferric Uptake Regulator (Fur) in Regulation of *Helicobacter pylori* Iron Uptake

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ABSTRACT

Background. Availability of the essential nutrient iron is thought to vary greatly in the gastric mucosa, and thus the human gastric pathogen *Helicobacter pylori* requires regulatory responses to these environmental changes. Bacterial iron-responsive regulation is often mediated by Ferric Uptake Regulator (Fur) homologs, and in this study we have determined the role of *H. pylori* Fur in regulation of *H. pylori* iron uptake.

Methods. Wild-type *H. pylori* and *fur* mutant derivatives were compared after growth in iron-restricted and iron-replete conditions. Iron-uptake was measured using ^{55}Fe-labeled iron, whereas gene expression was monitored at the transcriptional level using Northern hybridization and *lacZ* reporter gene fusions.

Results. Iron-uptake and total cellular iron content were approximately five-fold increased in the *fur* mutant compared with the wild-type strain, which indicated that in the *fur* mutant iron-uptake is not repressed by excess iron. A comprehensive screening of all *H. pylori* genes encoding putative iron-uptake proteins indicated that some of these *H. pylori* genes are constitutively expressed, while others are iron- and Fur-regulated.

Conclusions. Iron uptake in *H. pylori* is in part differently regulated compared with other bacteria, since in *H. pylori* some iron-uptake systems are constitutively expressed. However, other iron uptake systems of *H. pylori* display the iron- and Fur-mediated repression that is common in bacteria. Taken together, this Fur-mediated modulation of iron-uptake capacity may be a specific adaptation to the conditions in the human stomach, where iron starvation and iron overload can be encountered in relatively short time intervals.

Keywords. Iron uptake, transcription, gene regulation, Fur.

Iron is an essential element for almost all living organisms, as it is a cofactor required for activity of many enzymes, and acts as a catalyst in electron transport processes. However, in the presence of oxygen, iron catalyzes the formation of toxic oxygen radicals [1]. Therefore intracellular iron homeostasis is of critical importance to all cells, and in most bacteria this is mediated by the Ferric Uptake Regulator protein Fur [2]. This repressor down-regulates transcription of iron transport systems when the intracellular concentration of Fe^{2+} exceeds a certain level. The iron-responsive regulation is mediated by iron-dependent binding of the Fur protein to conserved DNA sequences (Fur boxes) located in the promoters of iron-regulated genes [2]. Fur homologs are present in both gram-negative and gram-positive bacteria, and besides their functions in iron homeostasis they are also involved in the regulation of additional metabolic processes, for example oxidative stress defense or acid resistance [2–4].

Helicobacter pylori is a gram-negative, microaerophilic bacterial pathogen, which colonizes the mucosal layer overlying the gastric epithelium

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of the human stomach. The presence of *H. pylori* in the gastric mucosa is associated with chronic active gastritis, which can develop in peptic ulcer disease and atrophic gastritis, an important precursor of gastric cancer [5]. Approximately half of the world's population is colonized by *H. pylori*, and the infection is therefore considered an important public health problem, with serious economic consequences [5].

In its natural niche, *H. pylori* is thought to encounter great variations in the iron concentration in the human stomach. Significant amounts of iron and heme compounds can be released from food by peptic degradation, or from gastric epithelial cells as a result of the *H. pylori*-mediated inflammatory response, while periods of iron-restriction may also be encountered since lactoferrin chelates iron at the mucosal surface [6]. The bioavailability of iron also depends on the oxidation state, which in turn is affected by the pH and oxygen tension of the environment [7]. Since *H. pylori* is capable of long-term colonization of the gastric mucosa, the bacterium is clearly able to respond to important environmental changes like iron availability. The *H. pylori* genome sequence contains a multitude of genes predicted to function in iron uptake [8–10]. However, experimental evidence for their function in iron transport is lacking, except for the FeoB ferrous iron transporter [11].

As most bacteria, *H. pylori* expresses a Fur homolog, which functions as an iron-dependent transcriptional repressor [12–15]. Recently it was demonstrated that Fur binds to the promoters of the iron-repressed HP0876 *frpB* gene [14] and the iron-induced *pfr* gene [13,15]. In this study we have established the role of the *H. pylori* Fur protein in regulation of iron uptake, by determining the effects of *fur* mutation in *H. pylori* on rates of iron uptake and on transcription of the comprehensive set of iron uptake genes of *H. pylori*.

Methods

Bacterial Strains, Plasmids, Media and Growth Conditions.

H. pylori strains used in this study were the wild-type strains 1061 [16], 26695 [8], NCTC 11638 (National Collection of Type Cultures), and their respective isogenic *fur* mutants [3,13,17]. *H. pylori* strains were routinely cultured on Dent agar [18], consisting of Columbia agar supplemented with 7% saponin lysed horse blood, 0.004% triphenyltetrazolium chloride (Sigma, St Louis, MO) and Dent Selective Supplement (Oxoid, Basingstoke, UK), at 37°C under microaerophilic conditions (10% CO_2, 5% O_2 and 85% N_2). Broth cultures were grown in Brucella Broth (Difco BD, Sparks, MO) supplemented with 3% Newborn Calf Serum (Gibco Life Techn., Breda, NL) (BBN). $FeCl_3$ (ACS quality) and desferal (deferoxamine mesylate) were purchased from Sigma, filter sterilised and used at the indicated concentrations. Iron-restriction was achieved by supplementing BBN with desferal to a final concentration of 20 μM, whereas iron-replete conditions were achieved by supplementing desferal-treated BBN with $FeCl_3$ to a final concentration of 100 μM. *E. coli* strains were grown aerobically in Luria-Bertani medium at 37°C [19]. For antibiotic selection, growth media were supplemented with ampicillin, kanamycin, or chloramphenicol to final concentrations of 100 μg/ml, 20 μg/ml and 10 μg/ml, respectively.

Iron Transport and Whole-Cell Iron Content Analysis

The uptake rates of ferrous iron and ferric iron were determined using ^{55}Fe as described previously [11]. For uptake assays, ferric iron was obtained by diluting $^{55}FeCl_3$ ten-fold in 1 M sodium citrate, while ferrous iron was obtained by tenfold dilution of $^{55}FeCl_3$ in 1 M ascorbate. Analysis of whole cell iron content was performed using atomic absorption spectroscopy as described previously [11].

Recombinant DNA Techniques

Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA) and Promega (Madison, WI), and standard protocols were used for manipulation of DNA and transformation of *E. coli* [19] and *H. pylori* [20]. Plasmid DNA was prepared using Qiaprep spin columns (Qiagen, Valencia, Spain). PCR was carried out using *Taq* polymerase (Promega).

RNA Hybridization

RNA was isolated from bacteria grown in iron-restricted or iron-replete conditions using RNeasy spincolumns (Qiagen) or Trizol (Gibco) according to the manufacturer's instructions. RNA was directly transferred to nylon membranes (Roche, Basel, Switzerland) using a Bio-Dot microfiltration

Table 1 Genes and their proposed functions, and corresponding oligonucleotides used in this study

Gene	Number[a]	(Proposed) Function	Forward primer (5′ → 3′)	T7-tagged reverse primer (5′ → 3′)[b]
*fecA*1	HP0686	Outer membrane siderophore receptor	CTCGCACGGTGATTTCTAAC	T7-TTAGGGCCATAACGCACGCT
*fecA*2	HP0807	Outer membrane siderophore receptor	AAGCTCTCGCACGGTGATTT	T7-AAGTGTTAGGGCCGTATTGG
*fecA*3	HP1400	Outer membrane siderophore receptor	GATTACCGCGCCTAAGAGTT	T7-CTGCCTCCACCCTTGATCAC
*frpB*1	HP0876	Outer membrane siderophore receptor	TCAACGCCAAAGCAATGAAG	T7-GGCTCTTTATCGGTCTGTGT
*frpB*2/3	HP0916/5[c]	Outer membrane siderophore receptor	AGAAGGCAAGCCAACCAAAT	T7-CCCACTTATGGCGTAGTCTT
*frpB*4	HP1512	Outer membrane siderophore receptor	AGCCGTCTCTTAAGGGTAAC	T7-TCGCTATTGCTTGGATCTTG
*ceuE*1	HP1562	Iron-transport periplasmic binding protein	AAGTGCCTGCCATGCTTAAT	T7-CTCTGCAATCGTTGTCTCTT
*ceuE*2	HP1561	Iron-transport periplasmic binding protein	ACTTGGG/TAGCTTTGCAGAA	T7-GCTTGAGCGTCAATATCTTC
fecD	HP0889	Cytoplasmic membrane permease	ATAGCGGTAGTGGAGTCTAA	T7-CGCAACAGCACCGGGTTAAT
fecE	HP0888	Cytoplasmic membrane ATPase	GCGCTTGGATTTAGAGTCCT	T7-TGGCAACCAAATTCGGATCA
feoB	HP0687	Cytoplasmic membrane Fe^{2+} transporter	ACTTACGCGCTCAATGACTT	T7-AGCCTGACAATTTCGTCTAA
pfr	HP0653	Ferritin iron storage protein	N/A[d]	N/A
nap	HP0243	Bacterioferritin/DNA-binding protein	TGCATAAAGCCACTGAAGAA	T7-TCATCCGCATAAGTTACGGT

[a]Gene number in the *Helicobacter pylori* 26695 genome sequence.
[b]Primers contained a 5′-extension with T7 promoter sequence (5′-ctaatacgactcactatagggaga-), for the creation of an antisense RNA probe.
[c]HP0916/0915 are two separate ORFs in *H. pylori* 26695, but comprise one ORF in *H. pylori* J99 [8,9].
[d]Probe was prepared using the SP6 promoter on plasmid pPFR1 [22].

Table 2 Transcriptional *lacZ* fusions of *Helicobacter pylori frpB* and *fecA* OM receptor genes

Gene[b]	Position promoter[c]	β-galactosidase[a] activity in wildtype strain[d]	β-galactosidase[a] activity in *fur* mutant[d]
*fecA*1 (HP0686)	738291–736905	14 ± 4	106 ± 18*
*fecA*2 (HP0807)	863754–862623	1 ± 1	40 ± 26*
*fecA*3 (HP1400)	1460556–1461748	256 ± 59	262 ± 24
*frpB*1 (HP0876)	926943–927679	2 ± 2	50 ± 5*
*frpB*2 (HP0916)	974334–973127	10 ± 3	25 ± 6*
*frpB*4 (HP1512)	1584242–1585207	10 ± 4	14 ± 4

[a]β-galactosidase activity is expressed in Miller units [19]. Asterisks indicate a significant difference in β-galactosidase activity in the *fur* mutant strain, when compared with the activity in the wild-type strain ($p < .01$, Student's *t*-test, n = 5–7).
[b]HP gene numbers corresponding to the *H. pylori* 26695 genome sequence [8].
[c]Position of duplicated fragment fused to the pBW *lacZ* gene, corresponding to the *H. pylori* 26695 genome sequence [8].
[d]*H. pylori* strains used were 1061 [16] and its isogenic *fur* mutant [3,17].

apparatus (Bio-Rad, Hercules, CA) [17,19]. Following transfer, RNA was covalently bound to the membrane by cross-linking with 0.120 J/cm² UV light of 254 nm wavelength. RNA samples were normalized based on 16S and 23S rRNA band intensities.

Internal fragments of target genes were PCR amplified with primers listed in Table 1. The resulting PCR fragments, which were all of a size between 200 and 400 bp, contained a T7 promoter sequence and were used for the production of antisense RNA probes labeled with digoxigenin by in vitro transcription using T7 RNA polymerase (Roche) [17]. Northern hybridization and stringency washes were performed at 68°C, and bound probe was visualized with the DIG-Detection Kit (Roche) and the chemiluminescent substrate CPD-Star (Amersham Pharmacia, Roosendaal, NL).

Construction of Transcriptional lacZ *Fusions in* H. pylori

DNA fragments containing the predicted promoter regions of all *fecA* and *frpB* genes in the *H. pylori* 26695 genome were amplified by PCR using primers designed from the 26695 genome sequence. The exact position of the DNA fragment is given in Table 2. The PCR fragments were cloned into the unique *Bgl*II site upstream of the promoterless *lacZ* gene of vector pBW [17,21], and the resulting plasmids were transformed to *H. pylori* 1061 and 1061 *fur* as described previously [20], resulting in kanamycin-resistant *H. pylori* strains. β-galactosidase activity of these strains was determined in lysates from freshly sonicated cells as described previously [17,19], and β-galactosidase activity was expressed in Miller units [19].

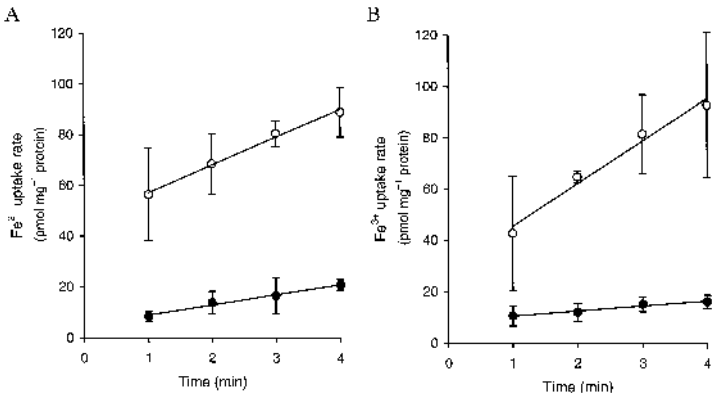

Figure 1 Iron uptake is increased in a *Helicobacter pylori fur* mutant. Rates of ferrous and ferric iron uptake in *H. pylori* wild-type and *fur* mutant were determined after transport was initiated with $^{55}Fe^{2+}$ (A) or $^{55}Fe^{3+}$-dicitrate (B) to cell suspensions of *H. pylori* NCTC 11638 wild-type (●) and *fur* mutant (○) cells. The graphs show the average of results of three independent experiments.

Results

Effect of the fur *Mutation on Iron Uptake and Whole Cell Iron Content*

The effect of the *fur* mutation on ferrous and ferric iron uptake rates of *H. pylori* was studied using ^{55}Fe in wild-type and *fur* mutant cells. Uptake rates of ferrous iron and ferric iron uptake were clearly increased in the *fur* mutant when compared with the wild-type strain (Figure 1). The increase was approximately four-fold for ferrous iron and six-fold for ferric iron, indicating that repression of iron uptake was absent in the *fur* mutant. Similar results were obtained with the *H. pylori* strain 26695 and its isogenic *fur* mutant (data not shown). Increased iron uptake as a result of *fur* mutation resulted in an increase in the whole cell iron content of the *fur* mutant (1.1074% iron of total dry weight) compared with the wild-type strain (0.2986% iron of total dry weight).

Transcriptional Analysis of H. pylori *Iron Transport and Storage Systems*

The *H. pylori* 26695 genome sequence contains 13 genes encoding putative components of *H. pylori* iron transport or iron storage systems (Table 1) [8]. Antisense RNA probes of these 13 genes were created, and hybridized with RNA isolated from *H. pylori* wild-type and *fur* mutant cells grown in iron-restricted and in iron-repleted conditions. Iron-responsive and Fur-mediated regulation was identified by visually comparing intensities of hybridization signals in the wild-type and *fur* mutant cells grown under different iron conditions (Figure 2).

Transcription of the *fecA*1, *fecA*2, *frpB*1 and *frpB*2 genes was iron-repressed in wild-type cells, but derepressed in the *fur* mutant (Figure 2), indicating Fur-regulation of these genes. This confirms the previously reported Fur-mediated regulation of the *frpB*1 gene [14,15]. Surprisingly, transcription of the *fecA*3 and *frpB*4 genes was not affected by changes in either iron concentration or the absence of Fur (Figure 2). Transcription of the *feoB* gene was also iron- and Fur-repressed (Figure 2). Transcription of the genes encoding the components of a cytoplasmic ABC-transporter (*ceuE*1, *ceuE*2, *fecD* and *fecE* genes) was not repressed by iron, and also not affected by the *fur* mutation (Figure 2). As controls we also hybridized the RNA samples with probes specific for the iron storage genes *pfr* and *napA* [15,22–24]. The transcriptional patterns obtained corresponded with those reported previously: transcription of *pfr* was iron-induced, Fur-dependent (Figure 2) [13,15], whereas transcription of *napA* was not affected by iron or the *fur* mutation (Figure 2) [24]. Results obtained using slotblotted RNA were confirmed by Northern hybridization (data not shown), although only mRNA species smaller than 1 kb remained intact. Especially *fecA* and *frpB* mRNAs were prone to degradation, resulting in smears with the maximum size of the hybridizing RNA smear of the predicted size based on analysis of the *H. pylori* genome sequence [8].

Regulation of fecA *and* frpB *Genes is Mediated via their Respective Promoter Regions*

Chromosomal transcriptional *lacZ* fusion [17,21] was used to confirm the transcriptional regulation demonstrated by RNA hybridization and to locate the corresponding promoter regions. These transcriptional fusions would also allow us to determine whether the observed regulation results from true transcriptional regulation (changes in de novo mRNA synthesis), or altered

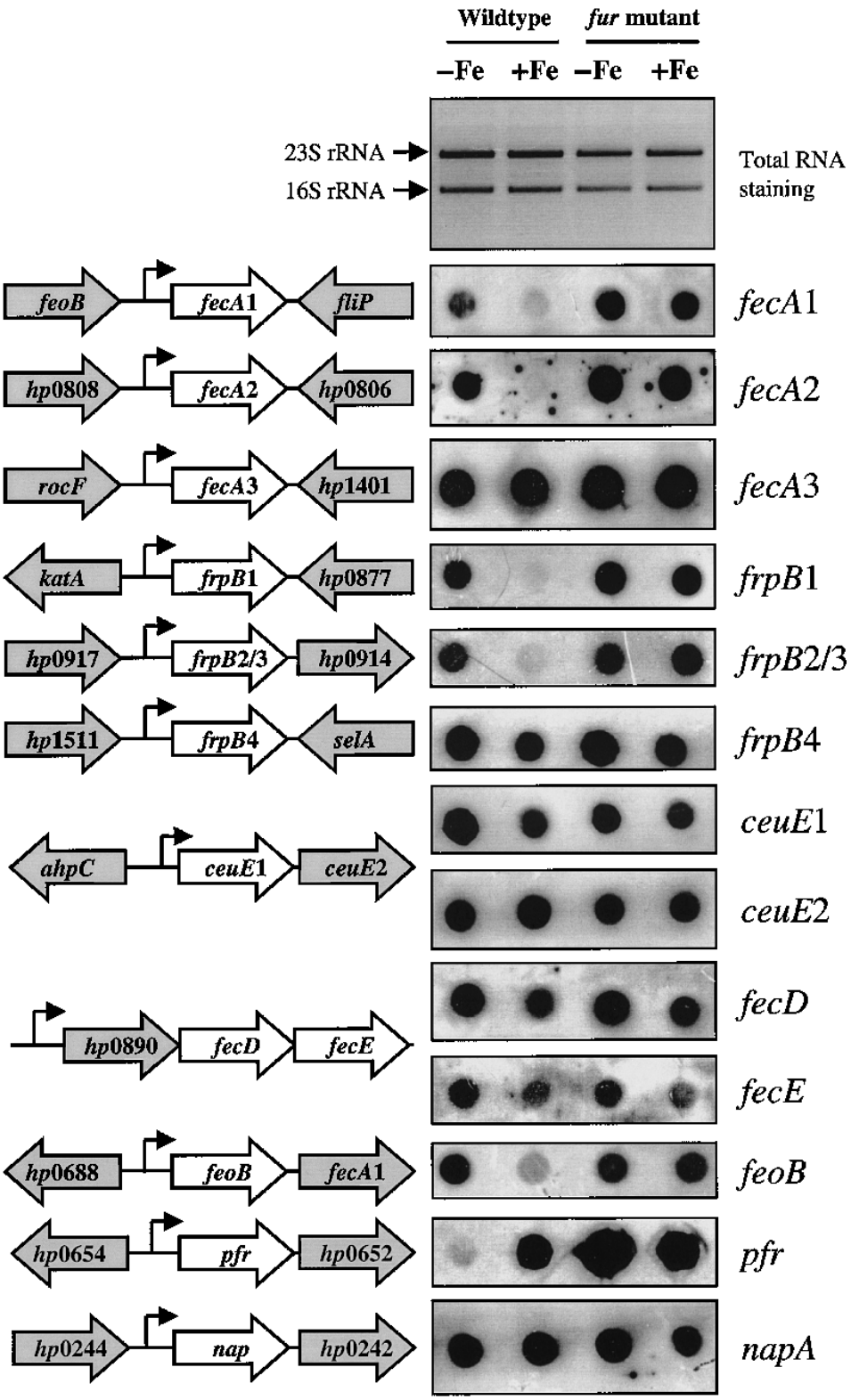

Figure 2 Transcriptional analysis of *Helicobacter pylori* 26695 iron transport and storage genes. Spotblot hybridization with probes specific for 13 putative iron acquisition and iron storage genes, using RNA purified from *H. pylori* wild-type and *fur* mutant cells grown in iron-restricted (–Fe) and iron-replete (+Fe) conditions. Probes used are indicated on the right, transcriptional organization and predicted location of the promoter of the corresponding gene is indicated on the left. Staining of transferred RNA by methylene blue is included for comparison of RNA amounts.

mRNA stability. The intergenic regions preceding all *fecA* and *frpB* genes were amplified by PCR, cloned in the correct orientation in front of the promoterless *lacZ* gene of pBW and transformed into *H. pylori* 1061 and 1061 *fur*. Integration of the pBW derivatives into the *H. pylori* chromosome by single homologous recombination leads to kanamycin resistance [17,21] and the duplication of the inserted DNA fragment, with one copy of the promoter fused to the promoterless *lacZ* gene and the other copy still preceding the intact gene [17,21]. As shown in Table 2, the β-galactosidase activities reflected the patterns of regulation shown by RNA hybridization; expression of the *fecA*1, *fecA*2, *frpB*1 and *frpB*2 *lacZ* fusions is repressed in *H. pylori* 1061, whereas in the absence of Fur expression is significantly increased (Table 2). In contrast, expression of the *fecA*3 and *frpB*4 *lacZ* fusions is not significantly affected by the *fur* mutation (Table 2).

Discussion

The varying and harsh conditions predicted to occur in the gastric mucosa have necessitated the development of adaptive mechanisms for *H. pylori*, and these are often mediated via gene regulation. Based on analysis of the complete genome sequences, it was predicted that *H. pylori* has a relatively limited capacity for gene regulation [8–10]. There are only three sigma factors, and homologs of bacterial global regulators such as RpoS, OxyR and SoxRS are absent [8–10]. One of the few global regulators present in *H. pylori* is the Fur protein [13,14,17,25]. The best studied function of Fur in bacteria is its control of iron transport [2], but in *H. pylori* Fur not only regulates iron uptake (this study and [14]), but also regulates iron storage [13,15], modulates urease expression in response to nickel [17], and is required for full acid resistance of *H. pylori* [3].

The transcriptional analysis of iron transport and iron storage genes suggests that *H. pylori* expresses iron transport systems in iron-replete conditions, but when exposed to iron-restriction, expresses additional iron transporters. This is in contrast to other bacteria, which only express iron uptake proteins when exposed to iron-restriction, since derepressed uptake of iron often leads to toxicity [1]. Confirmation for the mRNA studies was obtained using genomic transcriptional *lacZ* fusions in *H. pylori* strain 1061 [17,21] (Table 2). Since the use of the pBW-system is currently restricted to *H. pylori* strain 1061 [21], this also confirms that the transcriptional patterns are conserved between two different *H. pylori* strains, and are possibly also present in other *H. pylori* strains.

On the basis of our transcriptional analysis, the complement of genes expressed by *H. pylori* under iron-replete and iron-restricted conditions is summarized in Figure 3. In iron-replete environments, *H. pylori* expresses a single FecA and FrpB outer membrane protein, both CeuE periplasmic binding proteins, the FecDE ABC transporter proteins, the Pfr ferritin and the NapA protein. In iron-restricted conditions, all putative iron transport proteins are expressed, including the remaining two FecA and two FrpB outer membrane proteins, and the FeoB protein. Conversely, expression of the Pfr ferritin is decreased upon iron-restriction [13,15,24]. This model is consistent with the low but constitutive levels of iron transport in the wild-type strain, and the increased rates of iron transport (Figure 1) and increased whole cell iron content of the *fur* mutant. It may be possible to utilize the constitutive iron transport of *H. pylori* for the development of new antimicrobials, based on 'trojan-horse' noniron metalloporphyrins [26], but this requires further studies.

Compared with other bacteria, *H. pylori* can only utilize a relatively limited number of iron sources. All *H. pylori* strains tested can use ferric dicitrate, heme compounds and inorganic iron, while some strains might be able to utilize lactoferrin or transferrin [11,27–29]. Inconclusive results on *H. pylori* siderophore synthesis have been attributed to the ferric reductase activity of *H. pylori*, which is probably mediated via riboflavin synthesis [30]. Interestingly, *H. pylori* is much more sensitive to ferric dicitrate than to $FeCl_3$ [11,22]. This is consistent with the expression of *H. pylori* iron uptake systems as inferred from the transcriptional analysis (Figures 2 and 3). Ferric dicitrate is probably transported by the different components of the *H. pylori* Fec system, which can be composed of the constitutively expressed FecA3, CeuE and FecDE proteins, and their expression at moderate ferric-dicitrate concentrations may already lead to high intracellular iron concentrations, with associated toxicity [1].

In conclusion, the Fur protein plays a central role in iron-responsive gene regulation of *H. pylori*, since all iron-regulated genes identified so far

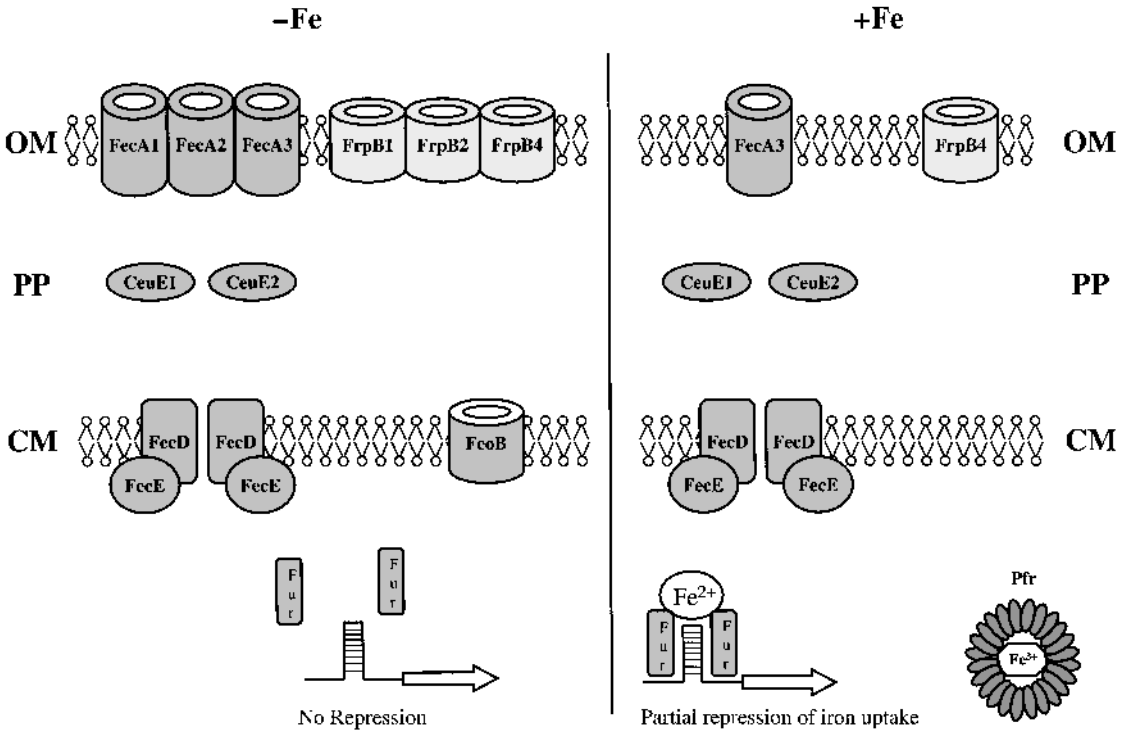

Figure 3 Schematic representation of regulation and predicted location of iron acquisition systems in *Helicobacter pylori*. –Fe: iron-restricted conditions; +Fe: iron-replete conditions; OM = outer membrane; PP = periplasm; CM = cytoplasmic membrane.

are regulated by Fur. However, in contrast to other bacteria, *H. pylori* does not down-regulate iron uptake in iron-replete conditions. Further studies are required to determine the role of Fur-mediated gene regulation in gastric colonization and inflammation, in identification of other members of the Fur regulon, and defining the binding sites of Fur in the promoters of its regulon.

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