Regulation of Ferritin-Mediated Cytoplasmic Iron Storage by the Ferric Uptake Regulator Homolog (Fur) of *Helicobacter pylori*

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Homologs of the ferric uptake regulator Fur and the iron storage protein ferritin play a central role in maintaining iron homeostasis in bacteria. The gastric pathogen *Helicobacter pylori* contains an iron-induced prokaryotic ferritin (Pfr) which has been shown to be involved in protection against metal toxicity and a Fur homolog which has not been functionally characterized in *H. pylori*. Analysis of an isogenic fur-negative mutant revealed that *H. pylori* Fur is required for metal-dependent regulation of ferritin. Iron starvation, as well as medium supplementation with nickel, zinc, copper, and manganese at nontoxic concentrations, repressed synthesis of ferritin in the wild-type strain but not in the *H. pylori* fur mutant. Fur-mediated regulation of ferritin synthesis occurs at the mRNA level. With respect to the regulation of ferritin expression, Fur behaves like a global metal-dependent repressor which is activated under iron-restricted conditions but also responds to different metals. Downregulation of ferritin expression by Fur might secure the availability of free iron in the cytoplasm, especially if iron is scarce or titrated out by other metals.

The gram-negative microaerophilic bacterial pathogen *Helicobacter pylori* colonizes the mucus layer of the human stomach (12, 20). Its hostile ecological niche has necessitated the development of regulatory mechanisms which allow the bacterium to survive unfavorable changes in the environment. Adaptation to the conditions in the gastric mucosa includes acquisition mechanisms that overcome a temporary lack of the metals iron and nickel. Iron is essential for maintaining the basic energy and redox metabolism, whereas nickel is an essential cofactor of urease, an important virulence determinant of *H. pylori* (21). However, as overacquisition of iron, nickel, and other metals is deleterious, the control mechanisms regulating the intracellular availability of these metals are of crucial importance.

Iron-responsive regulation in prokaryotes is usually mediated through the ferric uptake regulator (Fur) protein. Fur homologs downregulate the expression of genes involved in iron uptake when the cytoplasmic ferrous iron concentration increases, thus abolishing iron acquisition (24). Fur homologs using metal ions as cofactors have been identified in many bacterial species (7, 9, 13, 16, 19, 23, 30, 32, 33), and their regulatory functions range from regulation of metal uptake to more specific processes, like oxidative-stress defense, production of virulence factors, and acid resistance. That the Fur protein also activates transcription in response to iron was recently shown for the iron-induced superoxide dismutase SodB of *Escherichia coli* (11, 13).

In addition to regulation of iron uptake, the internal iron concentration within the cell can be modulated by the removal of free iron and manganese from the cytoplasm. This function is catalyzed by bacterial ferritins, which constitute a specialized intracellular compartment for the storage of iron in a nonreactive state and thus separate it from the biochemical processes in the cytoplasm. Knowledge about the role of Fur in the regulation of ferritin-mediated iron storage is very limited, as it has only been studied in *E. coli*, where ferritin expression was suggested to be positively regulated by the Fur protein (1). Iron-responsive regulation of Pfr has been observed in *H. pylori* (35, 36), and genetic analysis revealed that *H. pylori* possesses a Fur homolog (2, 6, 7, 14, 28). It was shown that in *E. coli*, the *H. pylori* Fur protein acts as an iron-dependent repressor of the Fur-regulated *E. coli* flu mutants and flu promoters (6, 14). The *H. pylori* ferritin protein Pfr is a member of the nonheme ferritin subfamily, all of which store iron in the inner space of a multimeric protein shell consisting of 24 identical subunits (4). The protein plays a substantial role in the storage of iron and protects the bacteria from metal toxicity (8, 10, 15). Ferritins thus catalyze a function which is the exact opposite of that of iron uptake systems, which increase the cytoplasmic iron concentration. In bacteria, the main functions of ferritins have been iron storage and protection against metal toxicity and oxidative stress (1, 3, 18, 25, 29, 34).

Although the structures and catalytic functions of bacterial ferritins are well known, data on their regulation in bacteria are limited (1, 4, 18). The accumulation of *H. pylori* Pfr in response to an increased iron concentration and its downregulation in response to iron starvation (8) indicated the presence of an iron-responsive regulatory network mediating iron homeostasis through modulation of cytoplasmic iron storage and release.

This study describes the role of *H. pylori* Fur in the regulation of ferritin-mediated iron storage. The observed Fur-dependent regulation of *H. pylori* Pfr was found to be affected by iron, as well as by nickel, zinc, copper, and manganese.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *H. pylori* strains NCTC11638 and G27 and mutant derivatives were routinely grown on blood agar in a microaerobic
RESULTS

Construction of a fur-negative mutant strain of H. pylori. To study the regulatory functions of the Fur homolog in H. pylori, an isogenic fur-negative mutant of strain NCTC11638 was constructed by marker exchange mutagenesis with plasmid pFUR3-CAT (Fig. 1). This resulted in an inactivation of fur by the insertion of a promoterless chloramphenicol resistance cassette. Analysis by Southern hybridization (Fig. 2) and by PCR (data not shown) confirmed correct replacement of the original wild-type fur gene by the interrupted version. The resulting H. pylori mutant strain, NCTC11638-FUR, was motile and positive for urease, catalase, and cytochrome oxidase.

Growth characteristics of the H. pylori NCTC11638 fur mutant. To determine whether the fur mutation resulted in growth deficiencies as observed in other bacterial species (32), the fur mutant and the parent strain were grown in liquid atmosphere as described previously (8). For determination of growth characteristics and responses to metals, bacteria were cultivated in brucella broth supplemented with 10% fetal calf serum (BBF; Gibco BRL). The total contents of iron, manganese, copper, zinc, and nickel in BBF medium were 20, 0.3, 0.5, 15, and 0.2 μM, respectively, as determined by atomic absorption mass spectrometry (26). Specific metal-enriched conditions were established by supplementation of BBF with chloride salts of iron (F2877; Sigma), manganese (M3634; Sigma), zinc (Z4875; Sigma), copper (C6641; Sigma), or nickel (N5756; Sigma) at 100 and 500 μM. Iron starvation was achieved by addition of the iron chelator desferrioxamine B (desferal; Sigma) at a concentration of 20 μM. Control cultures were supplemented with sodium chloride at 500 mM to exclude the effect of osmotic stress on regulation. For growth experiments, bacteria were cultured in liquid medium to an optical density at 600 nm of approximately 1 and subsequently diluted 1:100 in medium supplemented with desferal and/or different metals. All experiments were performed in triplicate and were repeated at least three times.

DNA and RNA techniques. The construction and basic characterization of the ferritin-negative H. pylori mutant strain G27-PFR1 (pfr::cat) are described elsewhere (8). Cloning of DNA was performed in E. coli according to standard protocols (5). Construction of the plasmid pFUR3-CAT carrying the H. pylori fur gene interrupted by a promoterless cat gene (Fig. 1A), is described elsewhere (6). Analysis of DNA by Southern hybridization and by PCR was performed as described earlier (6, 25).

To achieve marker exchange mutagenesis of the fur gene in H. pylori, plasmid pFUR3-CAT was transferred into H. pylori strain NCTC11638 by electroporation (17). Mutants carrying the promoterless cat gene from plasmid pFUR3-CAT inserted in the chromosomal fur gene were selected by growth on BBF agar containing chloramphenicol at a concentration of 10 mg/liter. Isolation of total RNA and Northern hybridization were performed according to a standard protocol (5) as described earlier for the fecA2 gene (14). Briefly, a digoxigenin (DIG)-labeled antisense RNA probe was produced by in vitro transcription of the pfr gene on plasmid pPFR1 (6) with SP6 RNA polymerase using the DIG RNA labeling kit (Roche Diagnostics). Hybridization and stringency washes were done at 68°C. Bound probe was detected with the DIG-labeling and detection kit using the chemiluminescent substrate CPD-Star (Roche Diagnostics).

Protein analysis. Measurement of protein concentration, electrophoretic separation of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein staining with Coomassie brilliant blue, and immunoblot analysis of proteins with the ferritin-specific antiserum AK198 were performed as described earlier (6). Bound antibodies were detected with a protein A-alkaline phosphatase conjugate. N-terminal Edman degradation was performed on protein bands blotted to polyvinylidene difluoride membranes in a protein-sequencing apparatus (model 477A/120A; Perkin-Elmer Applied Biosystems) according to the manufacturer’s instructions.

To quantify the Cat reporter protein produced in H. pylori strain G27-PFR1 by enzyme-linked immunosorbent assay (ELISA), bacteria grown in BBF media with different metal concentrations were harvested by centrifugation. Lysis and determination of the amount of Cat protein were performed using the Cat-ELISA system (Roche Diagnostics) according to the manufacturer’s recommendations. The amount of Cat was calculated from a standard curve prepared with purified Cat protein from E. coli and normalized to the amount of total protein.
media with various iron and nickel concentrations (Fig. 3). This analysis showed that, irrespective of the metal concentration, growth of the fur-negative mutant did not differ from that of the parent strain (Fig. 3). Under all growth conditions and at all sampling times in this study (see below), the growth of the mutant was identical to that of the wild-type strain (data not shown).

**Ferritin expression is derepressed in the H. pylori fur mutant.** To identify iron-regulated genes controlled by Fur, the parent *H. pylori* strain and the fur mutant were grown in iron-restricted and iron-sufficient BBF media, and their total protein profiles were compared (Fig. 4A). In the parent strain, a 19-kDa protein was expressed under iron-sufficient conditions but repressed under iron-restricted conditions (Fig. 4A). This 19-kDa protein was highly expressed in the fur-negative mutant under both iron-restricted and iron-sufficient conditions (Fig. 4A). The iron-regulated 19-kDa protein was identified as the ferritin protein Pfr by using the ferritin-specific antiserum AK198 (8) (Fig. 4B), and this was confirmed by N-terminal sequence analysis.

The involvement of Fur in the iron-dependent regulation of ferritin levels was further confirmed by Western blotting (Fig. 4B). The intensity of the ferritin protein band detected by the specific antiserum clearly decreased under conditions of iron starvation in the parent strain but not in the fur mutant (Fig. 5). This indicates that low iron levels lead to a Fur-mediated repression of pfr mRNA synthesis.

**Regulation of ferritin synthesis in response to metals.** To test whether *H. pylori* Fur regulates pfr transcription in response to other metals, the *H. pylori* parent strain and the fur mutant were grown in the presence of nickel, copper, manganese, or zinc at 100 or 500 μM, and Pfr expression was assessed by SDS-PAGE (Fig. 6). Ferritin expression was repressed by all metals in the wild-type strain but not in the fur mutant (Fig. 6). Regulation of ferritin synthesis was confirmed by Western blotting (not shown). The complete derepression of ferritin production in the fur-negative mutant strain in the presence of the tested metals indicated that Fur is involved in downregulation of ferritin synthesis mediated by other metals (Fig. 6). Supplementation of the medium with sodium chloride at the highest nickel concentration had no influence on production of the 19-kDa protein, excluding the influence of osmotic stress on regulation (not shown).

The inhibitory effects of nickel and of iron starvation on Pfr expression were quantitated using the promoterless cat cassette (Fig. 1) inserted in the pfr gene in the *H. pylori* strain G27-PFR1 (Fig. 7). Changes in pfr expression in response to iron starvation and to nickel were measured as changes in the levels of the Cat protein (Fig. 7). Iron starvation and nickel supplementation at concentrations of 0.1 and 1 mM reduced the Cat concentrations to 20, 50, and 9% of the levels detected under normal growth conditions, respectively (Fig. 7).

**DISCUSSION**

The *H. pylori* ferritin Pfr is a major component of iron storage, as well as iron distribution, in the cell (8, 10, 15). Under iron-rich conditions, which in the natural environment of *H. pylori* could be caused by free iron and heme compounds from nutrition or inflammation, ferritin protects the cell from iron toxicity. The same could be true for other metals. On the other hand, in response to iron starvation caused by the absence of nutrients or by the iron-binding functions of mucosal lactoferrin (22), repression of ferritin synthesis secures the availability of free iron in the cytoplasmic space for incorporation into essential iron-cofacted electron transport proteins (Fig. 8). For regulation of the free ferrous iron concentration in the cytoplasm, ferritins catalyze a reaction whose conditions of iron starvation in the wild-type strain but not in the fur mutant (Fig. 5). This indicates that low iron levels lead to a Fur-mediated repression of pfr mRNA synthesis.

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function is the exact opposite of that of iron uptake systems (Fig. 8). In contrast to the latter, ferritins lower the cytoplasmic concentration of ferrous iron by binding it and transporting it into the inner shell of the ferritin holoprotein. This function also lowers metal toxicity in other bacterial species, as shown for the E. coli ferritin FtnA (1, 29).

The concentration of free ferrous iron in the bacterial cytoplasm is mainly controlled by repression of iron uptake mediated by Fur homologs, by enhanced incorporation of iron into iron-cofactored proteins, and by the iron-binding properties of ferritins (Fig. 8). Therefore, combining regulatory functions for iron uptake and iron storage would be an advantage in maintaining iron homeostasis in the cell. The regulatory functions of Fur homologs in balancing the cytoplasmic metal concentration have been most extensively investigated for the regulation of metal uptake, whereas almost nothing is known about their role in the regulation of storage of iron and other metals (13).

Our previous studies indicated that H. pylori Fur is involved in regulation of iron metabolism and acts as a repressor of iron uptake systems in response to elevated levels of iron in the environment (6, 7, 31). In the induction of iron uptake systems, H. pylori Fur behaves similarly to Fur homologs of other bacteria (6, 14, 31), which are usually involved in differential gene regulation in response to changes in the environmental concentrations of metals (7, 9, 13, 16, 19, 23, 30, 32, 33). Evidence for binding of H. pylori Fur to the promoter regions of genes involved in iron uptake is provided by the presence of putative Fur binding sequences in some promoters of H. pylori genes predicted to be involved in iron uptake (28) and by isolation of putative H. pylori Fur-regulated promoters using a modified Fur titration assay (14). The comparison of Pfr expression in the wild-type and fur mutants performed in this study confirmed that H. pylori Fur is involved in metal-dependent regulation and, especially in combining modulation of both uptake and storage of iron, might have a wider range of functions in maintaining metal homeostasis than is known so far.

The regulatory role of Fur under iron-restricted conditions has not yet been studied, nor has its role in positive gene regulation (33). A possible influence of iron starvation on metal-dependent transcriptional repressor proteins was recently suggested for the zinc uptake regulator Zur of E. coli, for which activation by iron starvation was reported (23). In addition, the E. coli Fur protein has been demonstrated to contain two zinc atoms, and binding specificity is altered when these zinc atoms are removed (3).

The finding that H. pylori Fur is necessary for repression of ferritin synthesis in response to iron starvation suggests that Fur is active in the absence of iron and that it secures, via repression of ferritin, the availability of free iron in the cytoplasm under conditions where environmental iron is scarce. The fact that Fur-mediated regulation of Pfr expression occurs at the mRNA level provides evidence for DNA-binding activity of Fur in the absence of iron, but other regulatory mechanisms acting on mRNA stability, like those present in eukaryotes, cannot be excluded (27). The transcriptional regulation of ferritin synthesis and the repression in response to iron starvation and to nickel was well reflected by using the promoterless Cat gene as a reporter (Fig. 7), indicating that Cat is well suited for reporter gene analysis in H. pylori.

The increase in ferritin production observed in the fur mutant under conditions of iron starvation suggests that other regulators might be involved in regulation of iron storage. A direct interaction of Fur with ferritin transcription is supported by Cat-specific ELISA. The amount of Cat produced under iron-replete conditions in BBF medium was set at 100%. The values represent the means of three independent determinations. The error bars indicate standard deviations.
by the metal-dependent function observed. If iron is scarce, the metal/iron ratio in the cell could increase, and it can be suggested that under these conditions other metals become dominant over iron and activate Fur, resulting in repression of ferritin synthesis (Fig. 6 and 8). In this context, it can also be speculated that other metals compete with iron for incorporation in iron proteins and thus mimic iron starvation in the cytoplasm. Competition of nickel, manganese, and copper with iron is only possible at high concentrations of these metals, because their concentrations in BBF medium are about 50 to 100 times lower than the iron concentration (see Materials and Methods). Of the metals investigated, only zinc is present at a concentration which is similar to the concentration of iron.

In this context, the Fur-dependent repression of ferritin synthesis by nickel, copper, manganese, and zinc suggests that H. pylori Fur acts like a more global metal-dependent regulator. Multiple functions of Fur are not unexpected, because Fur homologs of other bacteria are involved in responses to iron and zinc and to peroxide stress, respectively (3, 9, 16). Because H. pylori possesses only a small set of regulatory proteins, and only one Fur homolog (2, 28), it would be reasonable if H. pylori Fur had multiple functions which would compensate for the absence of other regulators. The functions of H. pylori Fur in the regulation of iron storage seem to differ considerably from those of E. coli Fur, which regulates the ferritin homolog FtnA in a way opposite to that of H. pylori Pfr, as an E. coli fur mutant produces less FtnA than the parent strain.

In summary, H. pylori Fur acts like a metal-dependent regulator of Pfr-mediated iron storage. The question of whether regulation of ferritin synthesis is mediated directly by Fur or includes other proteins, as in eukaryotes (27), is the topic of ongoing studies focused on the functional DNA-binding properties of the Fur protein itself and on the investigation of member genes of the H. pylori Fur regulon.

FIG. 8. Schematic representation of iron metabolism in H. pylori under iron-rich, iron-restricted, and metal-rich conditions. The putative regulatory roles of Fur are indicated. Iron flux in the cytoplasm is indicated by the arrows. Free iron is indicated by the solid circles. The possible competitive inhibitory interactions between free iron and other metals (shaded circles) are indicated by a double arrow. The protein shell of ferritin is shown by the open circle. Repression of ferritin synthesis under conditions of iron starvation or metal overload, as well as release of ferritin-bound iron, is indicated by the dashed circle. Thick and thin arrows at the membrane indicate high and low transport, respectively. Arrows with two bars indicate inhibition of ferritin synthesis.

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