Iron-Responsive Regulation of the Helicobacter pylori Iron-Cofactored Superoxide Dismutase SodB Is Mediated by Fur

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Received 18 September 2004/Accepted 16 February 2005

Maintaining iron homeostasis is a necessity for all living organisms, as free iron augments the generation of reactive oxygen species like superoxide anions, at the risk of subsequent lethal cellular damage. The iron-responsive regulator Fur controls iron metabolism in many bacteria, including the important human pathogen Helicobacter pylori, and thus is directly or indirectly involved in regulation of oxidative stress defense. Here we demonstrate that Fur is a direct regulator of the H. pylori iron-cofactored superoxide dismutase SodB, which is essential for the defense against toxic superoxide radicals. Transcription of the sodB gene was iron induced in H. pylori wild-type strain 26695, resulting in expression of the SodB protein in iron-replete conditions but an absence of expression in iron-restricted conditions. Mutation of the fur gene resulted in constitutive, iron-independent expression of SodB. Recombinant H. pylori Fur protein bound with low affinity to the sodB promoter region, but addition of the iron substitute Mn2+ abolished binding. The operator sequence of the iron-free form of Fur, as identified by DNase I footprinting, was located directly upstream of the sodB gene at positions −5 to −47 from the transcription start site. The direct role of Fur in regulation of the H. pylori sodB gene contrasts with the small-RNA-mediated sodB regulation observed in Escherichia coli. In conclusion, H. pylori Fur is a versatile regulator involved in many pathways essential for gastric colonization, including superoxide stress defense.

The human gastric pathogen Helicobacter pylori is the causative agent of gastritis and peptic ulcer disease, and infection with H. pylori is associated with the development of adenocarcinoma of the distal stomach (20, 34). About one-half of the world’s population is infected with H. pylori, making it a very successful pathogen. This bacterium colonizes the mucus layer overlaying the gastric epithelial cells, and in this niche it is exposed to hostile environmental conditions caused by the acidic pH and by an active immune response (34). The extraordinary success of H. pylori in its hostile niche is indicative of very effective adaptation to these conditions.

The strong immune response of the host induces oxidative stress in H. pylori (34). In addition, reactive oxygen species like superoxides are also generated during bacterial respiration and metabolism (33). In view of the microaerophilic requirements of this organism and its low oxygen tolerance, it is not surprising that enzymes involved in the detoxification of reactive oxygen species are important H. pylori colonization factors. In H. pylori this is demonstrated by the inability of mutants with mutations in genes encoding components or regulators of detoxification enzymes to colonize the gastric environment in animal models (1, 3, 8, 10, 16, 17, 26, 27, 30, 41, 42).

Iron and oxidative stress defense are intimately linked, as iron potentiates the formation of toxic oxygen radicals through the Fenton and Haber-Weiss reactions (24). Therefore, the modulation of intracellular iron levels is of critical importance in oxidative stress defense (15). In many bacteria intracellular iron levels are controlled by the ferric uptake regulator Fur, which acts as a transcriptional repressor protein that displays iron-dependent binding to conserved DNA sequences (Fur boxes) located in the promoters of iron-regulated genes (15). In H. pylori, Fur displays differential binding to promoters depending on the presence or absence of the iron cofactor. As in other bacteria (15), the iron-complexed form of Fur in H. pylori binds to promoters of iron uptake genes, thus repressing iron uptake in iron-replete conditions (11, 39). Uniquely, the iron-free form of H. pylori Fur (apo-Fur) is also able to bind promoters, as was exclusively shown for the pfr gene, which encodes the H. pylori iron storage protein Pfr (4, 12, 40). Effectively, the binding of apo-Fur to the pfr promoter results in repression of ferritin expression in iron-restricted conditions (4, 40). In addition to regulation of iron metabolism, H. pylori Fur has been implicated in regulation of acid resistance (6) and oxidative stress resistance (3, 10, 16).

H. pylori expresses only a single superoxide dismutase (SOD), the iron-cofactored SodB protein (29, 32). Expression of SodB is essential for gastric colonization by H. pylori and is...
also required for growth under microaerophilic conditions (30). Recently, it was shown that expression of the sodB gene is subject to regulation in response to varying environmental conditions, including iron (14, 19). In this study we demonstrated that Fur mediates iron-responsive regulation of sodB expression in H. pylori by direct binding of apo-Fur to the sodB promoter region. To our knowledge, this is the first description of regulation of oxidative stress defense by apo-Fur.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and growth conditions.** H. pylori strain 26695 (35) and an isogenic fur mutant (6, 39) were routinely cultured on Columbia agar plates supplemented with 7% saponin-lysed horse blood and Dent selective supplement (Oxoid) at 37°C under microaerophilic conditions (10% CO₂, 5% O₂, and 85% N₂). Broth cultures were grown in brucella broth (Difco) supplemented with 3% newborn or fetal calf serum (Life Technologies, Breda, The Netherlands) (BBN) and were shaken continuously at 40 rpm. Iron restriction was achieved by supplementing BBN with desferal (deferoxamine mesylate) at 200 μM (39). Iron-replete conditions were achieved by supplementing desferal-treated BBN with ferric chloride (Sigma) at 0.52% (wt/vol) CHAPS, and 40 mM Tris, and incubated for 45 min at 37°C under microaerophilic conditions (10% CO₂, 5% O₂, and 85% N₂). Broth cultures were grown in brucella broth (Difco) supplemented with 3% newborn or fetal calf serum (Life Technologies, Breda, The Netherlands) (BBN) and were shaken continuously at 40 rpm. Iron restriction was achieved by supplementing BBN with desferal (deferoxamine mesylate) at 200 μM (39). Iron-replete conditions were achieved by supplementing desferal-treated BBN with ferric chloride (Sigma) at a final concentration of 20 μM (39). Iron-replete conditions were achieved by supplementing desferal-treated BBN with ferric chloride (Sigma) at a final concentration of 100 μM (39).

**Two-dimensional (2-D) polyacrylamide gel electrophoresis.** Cells were harvested by centrifugation. After removal of the supernatant, the pellets were washed with phosphate-buffered saline (pH 7.3), resuspended in 10 μl lysis buffer A, which contained 8 M urea, 4% (wt/vol) 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS), and 40 mM Tris, and incubated for 45 min at 37°C under microaerophilic conditions (10% CO₂, 5% O₂, and 85% N₂). Broth cultures were grown in brucella broth (Difco) supplemented with 3% newborn or fetal calf serum (Life Technologies, Breda, The Netherlands) (BBN) and were shaken continuously at 40 rpm. Iron restriction was achieved by supplementing BBN with desferal (deferoxamine mesylate) at 200 μM (39). Iron-replete conditions were achieved by supplementing desferal-treated BBN with ferric chloride (Sigma) at a final concentration of 100 μM (39).

**N2ase 1 footprinting.** N2ase 1 footprinting was performed using 440 pM of the DIG-labeled tagd-sodB intergenic region, which was mixed with of 0, 2.3, 4.6, 6.9, 9.2, or 16 μM Fur protein in DNase binding buffer (10 mM Tris-HCl, pH 8, 50 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.1% NP-40, 10% glycerol, 1 μg herring sperm DNA [12]). Reactions were carried out in the presence of 200 μM EDTA, and the mixtures were incubated for 30 min at 37°C. Subsequently, the DNA was digested with 0.25 U DNase 1 (Promega) for 1 min, and the reaction was stopped as described previously (12). Subsequently, the fragments were separated on a 7% polyacrylamide–8 M urea sequencing gel. Gels were blotted onto a nylon membrane (Roche), and this was followed by chemiluminescent detection of DIG-labeled DNA. To calculate the binding affinity of Fur for the promoter region of sodB, the autoradiograph was digitalized using a Canon CanoScan 5200F scanner at 300 dots per inch and analyzed by densitometry using the Kodak 1D image analysis software, version 3.5.

The transcription start site of the sodB gene of H. pylori 26695 was determined by primer extension analysis (13). Briefly, approximately 5 to 7 μg of total RNA of H. pylori wild-type strain 26695 and the isogenic fur mutant was incubated with 50 pmol of 5′-DIG-end-labeled primer SodB-R1 (Table 1) and avian myeloblastosis virus reverse transcriptase (Promega). Primer extension products were separated on a 5% polyacrylamide–8 M urea gel and blotted onto a nylon membrane (Roche), and this was followed by chemiluminescent DIG detection.

Electrophoretic mobility shift assay. Recombinant H. pylori Fur protein was produced in Escherichia coli and purified as previously described (38, 40). The sodB promoter region was PCR amplified with primers TagD-R1 and DIG-labeled SodB-R1 (Table 1), which flank the 374-bp tagd-sodB intergenic region. Electrophoretic mobility shift assays were performed with two independent isolations of recombinant Fur protein as described previously (38). Briefly, 22 pM of sodB promoter DNA was mixed with recombinant Fur protein at concentrations ranging from 0 to 4,500 nM. Protein and DNA were mixed in binding buffer (24% glycerol, 40 mM Tris-Cl, pH 8.0, 150 mM KCl, 2 mM DTT, 600 μg/ml bovine serum albumin, 50 ng herring sperm DNA) in a 20-μl (final volume) mixture and incubated at 37°C for 30 min. As indicated below, manganese chloride (MnCl₂) (Sigma) or EDTA was added to a final concentration of 200 μM. Samples were subsequently separated on a 5% polyacrylamide gel in running buffer (25 mM Tris, 390 mM glycine) for 30 min at 200 V. The gel was then blotted onto a nylon membrane (Roche Molecular Biochemicals), and this was followed by chemiluminescent detection of DIG-labeled DNA. To calculate the binding affinity of Fur for the promoter region of sodB, the autoradiograph was digitalized using a Canon CanoScan 5200F scanner at 300 dots per inch and analyzed by densitometry using the Kodak 1D image analysis software, version 3.5.

### RESULTS

Iron-responsive expression of SodB is mediated at the transcriptional level. Using DNA array-based transcriptional profiling, the sodB gene was recently identified as a member of a regulon comprising 15 H. pylori genes which display Fur-dependent, iron-induced transcription (14), similar to the previously described regulation of the pfr gene (4, 12). This regulatory pattern was further characterized for the sodB gene using Northern hybridization with RNA purified from H. pylori wild-type strain 26695 and an isogenic fur mutant, both grown in iron-restricted and iron-replete conditions (Fig. 1A). The sodB-specific probe hybridized to a single approximately 0.7-kb monocistronic mRNA (29). In the wild-type strain, this mRNA was detected after growth in iron-replete conditions (with Fe) and not after growth in iron-restricted conditions (without Fe), whereas the sodB mRNA was present in the fur mutant independent of iron availability (Fig. 1A) at levels even higher than those in the wild-type strain.

The transcription start site of the sodB gene in H. pylori strain 26695 was identified using primer extension analysis (Fig. 1B). There was only a single primer extension product, located at the A residue 21 bp upstream of the ATG start codon, and this matched the transcription start reported previously for H. pylori strain 60190 (29) despite the presence of three sequence differences in the sodB promoter sequences of the two strains. In accordance with the Northern hybridization data (Fig. 1A), the primer extension product could be detected.
in the wild-type strain only after growth in iron-replete conditions, whereas it was constitutively present in the fur mutant (Fig. 1B).

Iron- and Fur-responsive regulation of the sodB gene was confirmed at the protein level by two-dimensional protein gel electrophoresis (Fig. 1C). The SodB protein migrated according to its predicted molecular mass (~24 kDa) and pI (pI ~6.4) (18, 31), and it was positively identified using MALDITOF mass spectrometry. The SodB protein was expressed in the wild-type strain only after growth in iron-replete conditions, and it was absent in iron-restricted conditions. Conversely, in the fur mutant strain the SodB protein was expressed independent of iron availability (Fig. 1C).

**The Fur repressor mediates direct regulation of the H. pylori sodB gene.** To investigate whether the iron-responsive regulation of sodB expression is directly or indirectly mediated by Fur, we performed an electrophoretic mobility shift assay with the sodB promoter region, using recombinant H. pylori Fur protein. Manganese was used as a substitute for the iron cofactor, as it is more stable under the assay conditions used and has been shown to function like iron under in vitro binding conditions (15). In the absence of manganese, Fur caused a shift of mobility of the sodB promoter (Fig. 2A). Addition of manganese to the binding reaction mixture abolished the mobility shift (Fig. 2A), indicating that only the metal-free form of Fur (apo-Fur) is able to interact with the sodB promoter, which is consistent with the transcriptional regulatory pattern. The affinity of H. pylori apo-Fur for the sodB promoter region was low, and the $K_d$ value was ~260 nM at a DNA concentration of 22 pM (Fig. 2B and C).

Fur binds to an operator sequence located at positions −5 to −47 in the sodB promoter. The Fur binding sequence in the sodB promoter region was localized using DNase I footprinting (Fig. 3A). Addition of apo-Fur led to the protection of two regions in the sodB promoter, which were separated by a DNase I hypersensitivity site located at position −24 (Fig. 3A). The two protected regions span the area from nucleotide −5 to nucleotide −47 relative to the transcription start site, overlapping the −10 and −35 region of the sodB gene (Fig. 3B). While the regulatory pattern of sodB regulation resembles that of the pfr gene, the apo-Fur binding site in the sodB promoter exhibits only poor sequence homology (Fig. 3C) with the two high-affinity binding sites present in the pfr promoter region (12), which may explain the relatively low affinity of apo-Fur for the sodB promoter.

**DISCUSSION**

The versatility of iron in redox reactions has resulted in extensive biological use of iron as a cofactor of enzymes, but iron also potentiates the formation of reactive oxygen species like superoxides. Therefore, cells contain mechanisms for protection against iron-associated oxidative stress, and these mechanisms include detoxifying enzymes like catalases and superoxide dismutases, as well as proteins which carefully control cytoplasmic iron homeostasis by balancing the availability of free iron through control of iron acquisition and storage. As the central regulator of iron homeostasis in many bacteria, the Fur repressor often acts both directly and indirectly as a regulator of oxidative stress defense (2, 15, 33).
In *H. pylori*, Fur mediates iron-dependent repression of iron uptake systems, leading to expression of iron uptake proteins only when iron is required (11, 39). Conversely, Fur also mediates repression of iron storage systems in iron-restricted conditions, by repression of ferritin expression (4, 12). The switch between repression and induction of iron uptake is coupled to the iron availability in the cytoplasm; when iron is available, a Fur dimer forms a complex with ferrous iron and binds to Fur binding sequences (Fur boxes) in the promoters of iron uptake genes (15). However, the situation is not as clear for the switch in repression and induction of ferritin-mediated iron storage; while iron induction of ferritin expression is found in several bacteria, the role of Fur in this process is not universal. Since *H. pylori* colonizes the gastric mucosa, it is thought to encounter both severe iron restriction by lactoferrin and also periods of iron overload after release of iron from food sources (23). Thus, the ability to regulate genes in response to iron restriction and iron overload is an important feature thought to allow chronic colonization of the gastric niche.

SOD catalyzes the dismutation of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \), which is subsequently removed by catalase. *H. pylori* expresses a single SOD (SodB), which is cofactored by iron (29, 30, 32) and is essential for gastric colonization by *H. pylori* in an animal model (30). An absence of SodB leads to cessation of growth in microaerobic conditions and an increase in the DNA mutation rate, probably caused by oxygen radicals formed by iron via the Haber-Weiss and Fenton reactions (30). In *E. coli*, two cytoplasmic SOD species are present: the manganese-cofactored SodA protein and the iron-cofactored SodB protein. Expression of both the sodA and sodB genes is regulated by iron; in iron-restricted conditions only the SodA protein is expressed, whereas the SodB protein is expressed in iron-replete conditions (25). Iron-responsive repression of the sodA gene is mediated directly by Fur, while iron-responsive induction of the sodB gene is indirectly affected by Fur via the RyhB small RNA (22). Transcription of the RyhB small RNA is Fur dependent, and once transcribed, RyhB can bind to complementary sequences in the 5’ end of the sodB mRNA, blocking translation and making the mRNA unstable (21, 22).

Regulation of the oxidative stress defense in *H. pylori* has not been studied in detail, but recent studies indicated that expression of antioxidant genes is controlled by transcriptional regulation through an intricate regulatory network (3, 10, 16, 19, 40). This is exemplified by a compensatory increase in expression of the antioxidant protein NapA upon mutation of the antioxidant enzyme alkyl hydroperoxide reductase (AhpC) (26). In contrast to *E. coli*, regulation by small RNAs has not been described for *H. pylori*, but it could also not explain the regulation of the *H. pylori* ferritin gene *pfr* (4, 12) or the sodB gene (this study). As in *E. coli*, expression of both Pfr and SodB is iron induced, but in contrast to *E. coli*, the mRNA levels of *pfr* and *sodB* are constitutively high in the *fur* mutant (Fig. 1A) (4). This expression pattern of SodB suggested a direct role for apo-Fur in regulation of the sodB gene, as previously described for the *pfr* gene (12, 40).

Direct and sequence-specific binding of *H. pylori* apo-Fur to the *H. pylori* sodB promoter region was confirmed using an electrophoretic mobility shift assay and DNase I footprinting assays (Fig. 2 and 3). apo-Fur bound to the region overlapping the −10 and −35 promoter sequences present in the sodB promoter region.
The affinity of *H. pylori* apo-Fur for the *sodB* promoter was surprisingly low (*K_d = 260 nM*) compared to the affinity of metal-cofactored *H. pylori* Fur for the *amiE* promoter (~10 nM, calculated from the data of van Vliet et al. [38]). This low affinity may have biological significance. Genes involved in iron metabolism need to be tightly regulated to prevent iron surplus in the cell and therefore creation of Haber-Weiss and Fenton reactions. In contrast, the SodB protein is the only defense against superoxide stress in *H. pylori* (30), and thus its expression should not be repressed unless *H. pylori* encounters such severe iron restriction that even activating SodB enzyme is not feasible. Low- affinity binding of Fur to the *sodB* promoter is one way of achieving such regulation. 

The DNase I footprinting pattern obtained for the *sodB* promoter resembled those identified for the *pfr* promoter, as there was a DNase I hypersensitivity site between the two protected regions (Fig. 3). However, the operator sequence present in the *sodB* gene displayed only very limited sequence homology to those identified in the *pfr* promoter. The limited availability of binding sequences of apo-Fur currently precludes definition of a consensus sequence. This could be resolved by studying additional promoters which are regulated by apo-Fur, and several candidate promoters have been described recently (14), including the *hydABC* operon encoding the iron- and nickel-cofactored hydrogenase enzyme (28).

Despite its small genome, *H. pylori* is a highly successful colonizer of the human gastric mucosa, persisting lifelong unless it is eradicated by antibiotic treatment (7). The Fur protein, which is well known for its central role in iron homeostasis in bacteria, affects the expression of different pathways involved in normal metabolism, stress resistance, motility, and virulence (4, 8, 12, 36, 38, 39). In our study, we expanded the role of Fur in one of these aspects, the regulation of oxidative (superoxide) stress defense. Fur directly represses the expression of SodB when the iron cofactor is not available, thus not wasting valuable cellular resources. When iron is available, repression is terminated, allowing expression of iron-cofactored SodB in conditions in which the risk of formation of reactive oxygen species is high. This direct role of Fur contrasts with the indirect Fur-mediated regulation of iron-cofactored SOD in *E. coli* (22) and highlights the special aspects of *H. pylori* Fur compared to other eubacterial Fur proteins. In conclusion, this is the first description of a *sodB* gene that is directly regulated by apo-Fur, and thus the mechanism described here is a novel mechanism for regulation of expression of Fe-containing superoxide dismutases in prokaryotes.

**ACKNOWLEDGMENTS**

We thank Michael Hecker, in whose laboratories part of this work was carried out, Susanne Pohl for help with optimization of the 2-D procedure, and Dirk Albrecht for the protein identification using MALDI-TOF mass spectrometry.

This study was financially supported by grants 901-14-206 and DN93-340 from the Nederlandse Organisatie voor Wetenschappelijk Onderzoek to A.H.M.V.V. and J.G.K., respectively, and by grant Ki201/9-1 from the Deutsche Forschungsgemeinschaft to M.K.

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