Differential regulation of amidase- and formamidase-mediated ammonia production by the *Helicobacter pylori* Fur repressor*

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SUMMARY

The production of high levels of ammonia allows the human gastric pathogen Helicobacter pylori to survive the acidic conditions in the human stomach. H. pylori produces ammonia through urease-mediated degradation of urea, but it is also able to convert a range of amide substrates into ammonia via its AmiE amidase and AmiF formamidase enzymes. Here data are provided that demonstrate that the iron-responsive regulatory protein Fur directly and indirectly regulates the activity of the two H. pylori amidases. In contrast to other amidase-positive bacteria, amidase and formamidase enzyme activities were not induced by media supplementation with their respective substrates acrylamide and formamide. AmiE protein expression and amidase enzyme activity were iron-repressed in H. pylori 26695, but constitutive in the isogenic fur mutant. This regulation was mediated at the transcriptional level, via binding of Fur to the amiE promoter region. In contrast, formamidase enzyme activity was not iron-repressed, but was significantly higher in the fur mutant. This effect was not mediated at the transcriptional level, and Fur did not bind to the amiF promoter region. These roles of Fur in regulation of the H. pylori amidases suggests that the H. pylori Fur regulator may have acquired extra functions to compensate for the absence of other regulatory systems.
INTRODUCTION

The human pathogen Helicobacter pylori colonizes the mucus layer overlaying the gastric epithelium, thereby causing persistent gastritis which can develop into peptic ulcer disease and gastric carcinomas (1). H. pylori is able to survive and colonize this hostile acidic niche, aided by the expression of its acid resistance mechanisms (2,3). One of the major factors contributing to acid resistance of H. pylori is the production of ammonia by its urease enzyme, which is essential for gastric colonization in different animal models (4-7). However, the role of urease in gastric colonization extends beyond protection against gastric acid, since H. pylori urease mutants are still unable to colonize the gastric mucosa when gastric acid production is abolished with proton pump inhibitors (4).

Ammonia is a key component of bacterial nitrogen metabolism, as it is the preferred source of nitrogen for the synthesis of amino acids, pyrimidines and purines. Ammonia plays a central role in pathogenesis and metabolism of the important human pathogen H. pylori, since it not only serves as nitrogen source (8), but also contributes to epithelial cell damage and apoptosis (9,10), is involved in chemotactic motility (11), and is required for acid resistance (2,3). Urea is thought to be the main source of ammonia in the gastric environment, but H. pylori does have alternative pathways for the production of ammonia, via amino acid catabolism (12) and via the activity of its two paralogous amidases AmiE and AmiF (13,14).

Aliphatic amidase (AmiE; EC 3.5.1.4) and formamidase (AmiF; EC 3.5.1.49) catalyze the conversion of amide substrates to the corresponding carboxylic acid and ammonia (13,14).

Control of the intracellular nitrogen status is important for living organisms, and this can be mediated by several different nitrogen regulatory systems. These include the PII (GlnB) signal transduction protein and NtrBC two-component regulatory system, which are widespread throughout the bacterial kingdom (15), but alternative nitrogen regulatory systems
exist (16-18). Analysis of the *H. pylori* genome sequence did not reveal the presence of any of the aforementioned nitrogen regulatory proteins (19). The presence of nitrogen regulatory systems is likely though, since the activity of the different ammonia-producing enzymes seems to be balanced: absence of urease activity leads to higher amidase activity (13), whereas the combined absence of urease and arginase led to higher formamidase activity (14). Conversely, absence of arginase also led to alterations in the activity of the amino acid deaminases (12), and thus it is thought that the intracellular nitrogen status of *H. pylori* is controlled through yet unidentified regulatory systems.

Analysis of the genome sequence indicated that *H. pylori* has a relatively limited capacity for gene regulation, and thus it is possible that the few regulatory proteins present regulate multiple responses and metabolic processes (19). One well-characterized regulatory protein of *H. pylori* is the Ferric Uptake Regulator (Fur; footnote 1), which controls intracellular iron homeostasis via concerted expression of iron-uptake and iron-storage genes (20-23). Since Fur has also been implicated in acid resistance of *H. pylori* (24) as well as in regulation of urease expression (25), we hypothesized that Fur may also regulate expression of alternative ammonia-producing enzymes. Here we report that Fur regulates transcription, expression and activity of the AmiE amidase, and indirectly affects enzyme activity of the AmiF formamidase. Regulation of ammonia production via the iron-regulatory protein Fur may be an example of how *H. pylori* may compensate for its relatively small regulatory capacity.
EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, media and growth conditions

*H. pylori* strain 26695 (19) and its isogenic fur mutant (24) were routinely cultured on Dent agar (26), 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% CO₂, 5% O₂ and 85% N₂). Broth cultures were grown in Brucella Broth (Difco, Sparks, MD) supplemented with 3% Newborn Calf Serum (Gibco, Life technologies, Breda, The Netherlands) (BBN). Ferric chloride and desferal (defereroxamine mesylate) were purchased from Sigma, filter sterilised and used at the indicated concentrations. To determine the effect of amide substrate on *H. pylori*, BBN media were supplemented with acrylamide (Sigma) or formamide (Sigma) to final concentrations of 5 mM and 100 mM, respectively. 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 ferric chloride to a final concentration of 100 µM (20). *E. coli* DH5α MCR (Gibco) was grown aerobically in Luria-Bertani medium at 37°C (27). 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.

Protein analysis

*H. pylori* wild-type and fur mutant cells were grown in iron-restricted or iron-replete medium, centrifuged at 4000 × g for 10 min at 4°C, and concentrated in ice-cold PBS to a final OD₆₀₀ of 10. *H. pylori* cells were lysed by sonication for 15 sec on ice with an MSE Soniprep 150 set at amplitude 10. Protein concentrations were determined with the bicinchoninic acid
method (Pierce, Rockford, IL) using bovine serum albumin as standard. Samples containing approximately 30 µg of protein were separated by two-dimensional electrophoresis using a Multiphor II electrophoresis unit (Amersham Pharmacia, Roosendaal, The Netherlands). Isoelectric focussing was performed on 11 cm Immobiline DryStrips (Amersham Pharmacia) with a pH range of 3-10, and subsequently separated according to molecular weight (MW) on a ExcelGel SDS (Amersham Pharmacia) with an acrylamide concentration gradient of 12-14%. Proteins were subsequently stained with Coomassie Brilliant Blue (27), trypsin digested and analyzed by Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry using a Bruker Biflex III (Bruker Daltonics, Billerica, MA). Protein identification was performed using the Mascot program (28) and the OWL non-redundant composite protein sequence database (http://www.matrix-science.com).

**Amidase, formamidase and urease enzyme assays**

The enzymatic activity of urease, amidase and formamidase were determined in fresh *H. pylori* lysates by measuring ammonia production from hydrolysis of urea, acrylamide or formamide, respectively, by using the Berthelot reaction as described previously (13,14,25). The concentration of ammonia present in the samples was inferred from a standard NH₄Cl concentration curve. Enzyme activity was expressed as micromoles of substrate hydrolyzed per minute, per milligram of protein. Differences in enzyme activities were tested for their statistical significance with the Mann-Whitney U test.

**RNA hybridization**

RNA was isolated from bacteria grown in iron-restricted or iron-replete conditions using Trizol (Gibco), according to the manufacturer's instructions. RNA was separated on 2% formaldehyde-1.5% agarose gels in 20 mM sodium phosphate buffer and subsequently
transferred to nylon membranes (Roche, Basel, Switzerland) using standard protocols (25,27).

Following transfer, RNA was covalently bound to the membrane by cross-linking with 0.120
J/cm² UV light of 254 nm wavelength. RNA was visualized by methylene blue staining (25),
and RNA samples were normalized based on 16S and 23S rRNA band intensities. Internal
fragments of the amiE and amiF genes were PCR amplified with primers listed in Table 1.
The resulting PCR fragments contained a T7 promoter sequence on the non-coding strand,
and were used for the production of antisense RNA probes labeled with DIG by in vitro
transcription using T7 RNA polymerase (Roche). 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) (25).

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 (27) and H. pylori (26). Plasmid DNA was prepared using
Qiaprep spin columns (Qiagen, Valencia, CA). PCR was carried out using Taq polymerase
(Promega).

Gel retardation assay

Recombinant H. pylori Fur protein was purified from E. coli with the pASK-IBA Streptag
system (IBA, Göttingen, Germany) as described previously (29). DIG-labelled amiE and
amiF promoter fragments were amplified with primer combinations
AmiE-PrF/AmiE-PrR-DIG and AmiF-PrF/AmiF-PrR-DIG, respectively (Table 2), and
incubated with increasing concentrations of recombinant Fur for 30 min at 37°C in binding
buffer (20 mM Tris-Cl pH 8.0, 75 mM KCl, 1 mM dithiotreitol, 300 µg/ml BSA, 100 µM
MnCl$_2$, 12% glycerol). Samples were subsequently separated on a 5% polyacrylamide
(37.5:1) gel in running buffer (25 mM Tris, 190 mM glycine) for 30 min at 200V. The gel
was then blotted onto a nylon membrane (Roche), and DIG-labelled DNA was visualized
using the DIG-Detection Kit (Roche) and the chemiluminescent substrate CPD-Star
(Amersham Pharmacia).
RESULTS

Amidase enzyme activity is not substrate-inducible

In the amidase-positive bacteria *Pseudomonas aeruginosa* and *Mycobacterium smegmatis*, amidase activity is controlled by substrate availability via the AmiR-AmiC and AmiA proteins, respectively (30,31). These proteins mediate induction of amidase expression upon supplementation of growth media with the amide substrate (30,31). While orthologs of the corresponding amidase regulatory proteins are absent in *H. pylori*, inspection of the *H. pylori amiE* and *amiF* promoters indicated the presence of sequences resembling Furboxes, suggesting iron-responsive regulation of these genes (22,32).

In order to determine whether amidase and formamidase activity was substrate-inducible or iron-regulated, we determined the effect of substrate supplementation and varying iron-availability on amidase and formamidase activity of *H. pylori* strain 26695. The highest concentrations of amidase substrates that still allowed growth of *H. pylori* 26695 were 5 mM acrylamide and 100 mM formamide (data not shown). Unlike other bacterial amidases, supplementation with these concentrations of amide substrates did not result in induction of amidase or formamidase enzyme activity (Fig. 1). However, changing iron-availability had a pronounced effect on amidase activity, which was high in iron-restricted conditions, but was almost absent in iron-replete conditions (Fig. 1A). In contrast, formamidase activity was not changed in iron-restricted conditions when compared to iron-replete conditions (Fig. 1B). Thus we conclude that amidase and formamidase activity in *H. pylori* 26695 is not substrate-inducible, but that amidase activity is regulated by iron-availability while formamidase activity seems constitutive.

Amidase expression and activity, and formamidase activity is regulated by Fur
The AmiE (HP0294) protein was previously identified as a protein of approx. 45 kDa with a pI of 6.4 (33). A protein of similar molecular mass and pI was identified when comparing 2D-protein profiles for identification of Fur- and iron-regulated proteins of *H. pylori* 26695 (Fig. 2). Wild-type cells expressed this protein when grown in iron-restricted conditions, but not in iron-replete conditions. This iron-repression was absent in the *fur* mutant strain (Fig. 2), suggesting that iron regulation was mediated by Fur. Subsequent identification of the protein by mass spectometry confirmed that this iron- and Fur-repressed protein was indeed AmiE (13,14). Since the AmiF protein has not been identified on 2D-gels yet (33), we were unable to compare AmiF protein expression levels.

To assess whether the effect of Fur and iron on AmiE at the protein expression level was also present at the enzyme activity level, we determined amidase activity in lysates of *H. pylori* 26695 and its isogenic *fur* mutant, grown in iron-restricted and iron-replete conditions (Fig. 3). As control we also determined formamidase activity in both strains and medium conditions. Amidase activity displayed identical regulation as observed at the protein expression level: in wild-type cells amidase activity was high at iron-restricted conditions and absent in iron-replete conditions (*P* < 0.01), whereas in the *fur* mutant activity was always high, independent of iron-availability (*P* = 0.56; Fig. 3A). Surprisingly, formamidase activity was also affected by the *fur* mutation: formamidase activity did not differ between cells grown in iron-restricted and iron-replete conditions, but differed significantly between the wild-type and *fur* mutant cells (*P* < 0.02; Fig. 3B). In wild-type cells formamidase activity was low but present, whereas formamidase activity was increased almost threefold in the *fur* mutant (Fig. 3B). These results were reproduced with a second, independently constructed *H. pylori* 26695 *fur* mutant (data not shown), indicating that the increase in formamidase activity is not caused by a secondary mutation.
Fur mediates regulation of amiE, but not amiF at the transcriptional level

Regulation via iron and Fur is usually mediated at the transcriptional level (32). The observed iron- and Fur-responsive regulation of AmiE expression was indeed reflected at the mRNA level, as demonstrated by Northern hybridization (Fig. 4). There was no amiE mRNA detected in the wild-type strain under iron-replete conditions, but transcription of a 1 kb mRNA was clearly apparent in iron-restricted conditions. In contrast, in the fur mutant amiE mRNA was present irrespective of the iron-availability of the medium (Fig. 4). The effect of the fur mutation on formamidase activity is however not mediated at the transcriptional level, since the small changes in the levels of amiF mRNA observed on Northern hybridizations (Fig. 4) did not correlate with the changes in enzyme activity observed (Fig. 4).

Specific binding of Fur to the amiE promoter, but not to the amiF promoter

The Fur protein normally functions by metal-dependent binding to a binding sequence (Furbox) located in the promoter region of the regulated gene (32). Analysis of the sequence directly upstream of the amiE and amiF genes had already indicated the presence of putative Furboxes (Fig. 5A). To confirm that amiE and amiF transcription were indeed differentially regulated by Fur, we performed gel retardation assays using recombinant H. pylori Fur (29) and DIG-labelled amiE and amiF promoter regions. Addition of recombinant H. pylori Fur with the metal cofactor Mn²⁺ to the amiE promoter region shifted mobility of the amiE promoter, consistent with binding of Fur to this promoter (Fig. 5B). Gel retardation was dependent on the presence of the Mn²⁺ metal cofactor (not shown). To check sequence specificity, we also used an internal fragment of the amiE gene, whose mobility was not affected by Fur (not shown). Finally, as predicted from the Northern hybridization experiments, but despite the presence of Furbox-like sequence, mobility of the amiF promoter was not affected by Fur (Fig. 5B).
DISCUSSION

Many species of the genus Helicobacter colonize the acidic gastric mucosa of humans and animals, and in this respect they represent unique pathogens (1). Colonization is dependent on acid-resistance, and while this process is multifactorial, the production of high levels of ammonia is essential to allow initial infection as well as subsequent colonization. Acid resistance of *H. pylori* has long been considered to be solely based on unregulated production of large amounts of urease, but recent studies have shown that acid resistance of *H. pylori* is based on multifactorial, interactive and probably well regulated processes (3,25,34-36). In these processes metal-responsive regulatory proteins play an important role, with the NikR protein regulating urease expression (25,34) and the Fur protein regulating iron homeostasis, acid resistance (20-24) and amidase- and formamidase-mediated ammonia production (this study) (Fig. 6).

Under physiological conditions, the optimal pH for *H. pylori* growth lies between 4 and 6. As a result, the colonization pattern of *H. pylori* varies with the level of acid production within the host stomach, and these different colonization patterns are associated with different long-term outcomes of infection (37,38). As such, *H. pylori* acid resistance is very relevant for the clinical outcome of disease and may also offer clues for therapy. *H. pylori* produces large amounts of ammonia through urea degradation, and actually requires an acidic environment to survive in the presence of urea, due to alkalinization of the medium to toxic levels at neutral pH (39). The presence of alternative pathways for the production of ammonia is likely to have evolved for situations where either urea is not available, where ammonia production is required at neutral pH conditions, or when toxic concentrations of amides are encountered in the natural niche of *H. pylori*.

Amidase enzymes are often present in environmental bacteria, where they function in the degradation of toxic amides in the environment, and are of interest for waste disposal. The
function or natural substrate(s) of the *H. pylori* amidases are not yet known, and thus it is
difficult to predict their exact function in *H. pylori* metabolism. While it is difficult to envisage
high levels of amides being produced intracellularly in *H. pylori*, recent reports of possibly
toxic or carcinogenic concentrations of acrylamide in food have raised concerns for public
health (40). The acrylamide can be produced after Strecker degradation of asparagine or
methionine in the presence of dicarbonyl compounds via the Maillard reaction (41,42). Of
special interest in the gastric environment may be the route via methionine since this reaction
has a requirement for ammonia, as produced by *H. pylori* (41,42). Furthermore, although it is
possible that the amidases function in protection against toxic amides, our preliminary data
indicate that production of the AmiE amidase does not increase protection against toxic
concentrations of acrylamide in a disc assay (data not shown).

Regulation of amidase expression was so far only studied in *P. aeruginosa* and in *M.
smegmatis*, where amidase expression is induced upon supplementation with amide substrate
(30,31). We have demonstrated here that amidase and formamidase activity is not substrate-
induced in *H. pylori*, but that amidase activity is Fur- and iron-repressed. This unexpected type
of regulation may be explained by either a role for amidase in siderophore synthesis, or by a link
between amide-availability and iron-availability. Amidases like AmiE and AmiF can form
hydroxamates, via an acyl transferase reaction using hydroxylamide as acceptor molecule (14).
Hydroxamates are an important class of siderophores, and in siderophore-producing bacteria the
biosynthesis of siderophores is usually iron-regulated (43). *H. pylori* lacks orthologs of bacterial
siderophore biosynthesis genes (19), but may use amidase-mediated formation of hydroxamates
as an alternative route to produce siderophores. However, the toxicity of hydroxylamine makes it
unlikely that *H. pylori* is able to safely produce the quantities of hydroxylamine necessary to
scavenge sufficient iron from the gastric environment.

An alternative possibility is that there may be a link between the availability of iron and
amide substrates. Both the urease- and amidase enzymatic reactions lead to the production of ammonia, but while the urease reaction results in alkalinization of the environment (39), the amidase reaction is pH-neutral (13,14). Thus amidase-generated ammonia is probably not sufficient for acid-resistance of *H. pylori* (44), but may still be used to form urea through the previously suggested urea cycle of *H. pylori* (12,45), and thus amidase activity may be important when urea availability is low. Alternatively, since ammonia also plays an important role in nitrogen metabolism, the pH-neutral production of ammonia by both amidases may allow production of sufficient intracellular concentrations of ammonia without alkalinization of the cellular environment.

Finally, a coupling between iron-availability and substrate-availability is supported by studies on the function and secretion of the *H. pylori* vacuolating cytotoxin VacA (46,47). Firstly, the VacA protein has been suggested to function as a urea permease, promoting urea diffusion from epithelial cells (46). Secondly, VacA is present in outer-membrane vesicles which are thought to deliver pro-inflammatory proteins to the epithelial cells, but only in iron-replete conditions (47). Combined, this would result in high urea release in iron-replete conditions, but low urea release in iron-restricted conditions. It is under these conditions where urea availability is low that amidase activity may be an alternative source of ammonia, and as such make iron-repression of amidase physiologically relevant.

Surprisingly, the *amiE* and *amiF* genes were differentially regulated by Fur. The *amiE* gene is regulated at the transcriptional level by Fur, while the *fur* mutation only affects enzyme activity of AmiF, but not *amiF* transcription (Figs. 4, 5). The mechanism behind the increased formamidase activity in the *fur* mutant is currently unknown. We hypothesize that this increase may be due to the altered intracellular environment caused by the pleiotropic effects of the *fur* mutation, by changes in availability of a yet unknown enzyme cofactor, or by altered stability or conformation of the formamidase enzyme. We have also tested a
second, independent fur mutant in *H. pylori* strain 26695 which contains a promoterless chloramphenicol cassette in *fur* (23). This independent *fur* mutant also displayed derepressed amidase activity and increased formamidase activity (data not shown), thus excluding the possibility that the effect on formamidase activity result from a secondary mutation or polar effects of the antibiotic cassette inserted in the *fur* gene. The Fur protein showed specific binding to the *amiE* promoter, but not to the *amiF* promoter, despite both promoters having sequences resembling Furboxes (Fig. 5A). This again demonstrates the limitations of Furbox predictions that are based solely on sequence similarity (48).

In conclusion, we have identified a novel type of gene regulation for bacterial amidases, which is mediated by Fur at the transcriptional and enzyme activity level (for AmiE) and at the enzyme activity level (for AmiF). The diverse roles of the Fur regulatory protein in metabolic and pathogenic processes of *H. pylori* indicate that this bacterium is able to use several intricately linked mechanisms to survive and thrive in the gastric mucosa, and is able to sense and cope with the variable conditions and multiple stresses occurring there despite its relatively limited range of regulatory proteins.
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FOOTNOTES

1. The abbreviations used are: Fur: Ferric Uptake Regulator, AmiE: aliphatic amidase, AmiF: formamidase, BBN: Brucella Broth supplemented with 3% Newborn calf serum, PBS: Phosphate Buffered Saline pH 7.3, BSA: Bovine Serum Albumin, MALDI-TOF: Matrix Assisted Laser Desorption/Ionization-Time of Flight, DIG: digoxigenin, PCR: Polymerase Chain Reaction, mRNA: messenger ribonucleic acid, kb: kilobase, pI: isoelectric point
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Table 1 Oligonucleotide primers used in this study

| Primer name      | Sequence (5’ → 3’) |
|------------------|--------------------|
| Amid-F1          | AGTAGCAGCCCCAGATCTGT |
| Amid-R-T7        | ctaatacgactatataggagaGCACGATCTCACCCCTTATCA |
| Amid-PrF         | ACGCGCTATATGGCTTAGTGGAAGT |
| Amid-PrR-DIG     | ACTACCGCTACACCCACAGTATC |
| Form-F1          | TCAGTTTCTGTGCCAATTGTCA |
| Form-R-T7        | ctaatacgactatataggagaCTCAATGGGATTCCATGGGAATA |
| Form-PrF         | CACCCAGAAAGTAGCCACCAGGTC |
| Form-PrR-DIG     | CAATAGGGTTTGCCCATACTACCGAT |

a) Primer sequences were derived from the *H. pylori* 26695 genome sequence (19)
b) Primers contained a 5’-extension with T7 promoter sequence (in lowercase letters), for the creation of an antisense RNA probe (25).
c) Primer was labelled at the 5’ end with DIG, for use in gel retardation assays.
LEGENDS TO FIGURES

Figure 1

Amidase and formamidase activity in *H. pylori* is not substrate-inducible, but amidase activity is iron-repressed. (A) Amidase activity in *H. pylori* grown in iron-replete (+Fe) and iron-restricted (-Fe) BBN medium without (black bars) and with acrylamide (white bars) supplemented to a final concentration of 5 mM. (B) Formamidase activity in *H. pylori* grown in iron-replete (+Fe) and iron-restricted (-Fe) BBN medium without (black bars) and with formamide (white bars) supplemented to a final concentration of 100 mM. Graphs represent three independent experiments, error bars denote standard deviations. Statistical evaluations of the comparison of enzyme activities using the Mann-Whitney U test are given.

Figure 2

Iron-regulated expression of the *H. pylori* AmiE protein is mediated by Fur. Protein profiles of *H. pylori* 26695 wild-type and fur mutant cells, grown in iron-restricted (-Fe) and iron-replete (+Fe) conditions, were compared on 2D-protein gels. The relevant part of the protein gel is magnified for each gel, and the iron- and Fur-repressed AmiE protein is circled. The estimated molecular mass and pI are indicated.

Figure 3

Effect of varying iron-availability on activity of (A) amidase and (B) formamidase activity in *H. pylori* 26695 wild-type and fur mutant strains. Enzyme activities were compared in lysates of cells grown in iron-replete conditions (black bars) and iron-restricted (white bars) conditions, and their respective enzyme activities were determined. Graphs represent a minimum of five independent experiments, error bars denote standard deviations. Statistical
evaluation of the comparison of enzyme activities using the Mann-Whitney U test are given.

**Figure 4**

Differential effect of Fur on *amiE* and *amiF* transcription. RNA was isolated from *H. pylori* 26695 wild-type and *fur* mutant cells, grown in iron-restricted (-Fe) and iron-replete (+Fe) conditions, and subjected to Northern hybridization with *amiE* and *amiF*-specific probes. Top panel: Staining of transferred RNA for comparison of RNA amounts; middle panel: hybridization with the *amiE*-specific probe; lower panel: hybridization with the *amiF*-specific probe. rRNA species and hybridizing RNAs are defined on the right hand side.

**Figure 5**

The Fur protein binds specifically to the *amiE* promoter, but not to the *amiF* promoter. (A) Identification of putative Furboxes in the promoters of the *amiE* and *amiF* genes. The Furbox consensus sequence is given on the top, with the identified *amiE* and *amiF* sequences boxed. Residues identical to the Furbox consensus sequence are underlined. The distance of the putative Furboxes to the Ribosome Binding Site (RBS) and Start codon are also indicated. (B) Gel retardation assay of the *amiE* and *amiF* promoter regions and increasing amounts of recombinant *H. pylori* Fur. The unbound promoters are indicated by P_{amiE} and P_{amiF}, and the retarded fragment is indicated as Fur-P_{amiE}, respectively.

**Figure 6**

Schematic representation of the role of Fur in ammonia production of *H. pylori*, and the role of ammonia in *H. pylori* metabolism and virulence.
Figure 1

(A) Amidase activity (µmol acrylamide/min/mg protein) in (+Fe) and (-Fe) medium supplemented with amides.

(B) Formamidase activity (µmol acrylamide/min/mg protein) in (+Fe) and (-Fe) medium supplemented with amides.

- Unsupplemented medium
- Amide-supplemented medium

Statistical significance:
- P < 0.01
- P = 0.28
- P = 0.51
- P = 0.05
- P < 0.01
- P = 0.52
Figure 2

-Fe +Fe

Wild-type

fur mutant

MW ≈ 45 kDa

pI = 6.4
Figure 3

(A) Amidase activity (µmol acrylamide/min/mg protein) in 26695 fur strain compared to fur-Regulated conditions.

(B) Formamidase activity (µmol formamide/min/mg protein) in 26695 fur strain compared to fur-regulated conditions.

Iron-replete conditions
Iron-restricted conditions

$p = 0.35$
$p < 0.01$
$p = 0.56$
$p < 0.01$
$p < 0.01$
$p = 0.23$
Figure 4

Wild-type fur mutant
-Fe +Fe -Fe +Fe

23S rRNA
16S rRNA
amiE
amiF
Figure 5

(A)

| Consensus | RBS | Start |
|-----------|-----|-------|
| nATwATnATwATnATwATn | | |
| amiE | TCGCC | CACAATAATCATAATGATT | AAAGT <81 bp> AAGGAACATAATATG |
| amiF | CTCGC | AATAATTGTATTGTATT | GCGAC <11 bp> AAGGAGTTATTATG |

(B)

![Image of gel electrophoresis showing Fur regulation of Helicobacter pylori amidases](http://www.jbc.org/Downloaded)
Figure 6

Diagram showing the regulation of Helicobacter pylori amidases by Fur. Fur binds to Fe, activating Amidase, which converts NH₃ to Acid resistance. Fur also regulates Formamidase, which affects Tissue damage and Nitrogen metabolism.
Differential regulation of amidase- and formamidase-mediated ammonia production by the Helicobacter pylori Fur repressor

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