Differential Regulation of Amidase- and Formamidase-mediated Ammonia Production by the Helicobacter pylori Fur Repressor*

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Arnaud H. M. van Vliet‡‡, Jeroen Stooft‡, Sophie W. Poppelreurs‡, Stefan Bereswill†, Georg Homuth‡, Manfred Kist‡, Ernst J. Kuipers‡, and Johannes G. Kusters‡

From the ‡Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center Rotterdam, 3015 GD Rotterdam, The Netherlands, the †Department of Medical Microbiology and Hygiene, Institute of Medical Microbiology and Hygiene, University Hospital of Freiburg, D-79104 Freiburg, Germany, and the Institute for Microbiology, University of Greifswald, D-17487 Greifswald, Germany

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 medium 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 the 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 suggest that the H. pylori Fur regulator may have acquired extra functions to compensate for the absence of other regulatory systems.

The human pathogen Helicobacter pylori colonizes the mucous 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, because 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, because 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, because 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 (13) 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).

The control of the intracellular nitrogen status is important for living organisms, and this can be mediated by several different nutrient regulatory systems. These include the $P_{\text{II}}$ (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). An 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, because the activity of the different ammonia-producing enzymes seems to be balanced. The 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, the 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.

An 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), which controls intracellular iron homeostasis via concerted expression of iron-uptake and iron-storage systems.

§ To whom correspondence should be addressed: Dept. of Gastroenterology and Hepatology, Rm. L–455, Erasmus MC-University Medical Center Rotterdam, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands, Tel.: 31-10-4635944, Fax: 31-10-4632793; E-mail: a.h.m.vanvliet@erasmusmc.nl.

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\† To whom correspondence should be addressed: Dept. of Gastroenterology and Hepatology, Rm. L–455, Erasmus MC-University Medical Center Rotterdam, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands, Tel.: 31-10-4635944, Fax: 31-10-4632793; E-mail: a.h.m.vanvliet@erasmusmc.nl.

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\* The abbreviations used are: AmiE, aliphatic amidase; AmiF, formamidase; Fur, ferric uptake regulator; BBN, Brucella Broth supplemented with 3% newborn calf serum; BSA, bovine serum albumin; DIG, digoxigenin.
genes (20–23). Because 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 the 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 on amidase and formamidase activity of 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), and Dent-selective supplement (Oxoid, Basingstoke, United Kingdom) 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 (Invitrogen) (BBN). Ferric chloride and desferal (deferoxamine mesylate) were purchased from Sigma, filter-sterilized, 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 and 100 mM, respectively. Iron restriction was achieved by supplementing BBN with desferal to a final concentration of 100 μM (20). Escherichia coli DH5α MCR (Invitrogen) was grown aerobically in Luria-Bertani medium at 37 °C. For antibiotic selection, growth media were supplemented with ampicillin, kanamycin, or chloramphenicol to final concentrations of 100, 20, and 10 μg/ml, respectively.

Protein Analysis—H. pylori wild-type and fur mutant cells were grown in iron-restricted or iron-repleted medium, centrifuged at 4000 g for 10 min at 4 °C, and concentrated in ice-cold phosphate-buffered saline to a final A600 of 10. H. pylori cells were lysed by sonication for 15 s on ice with an MSE Soniprep 150 set at amplitude 10. Protein concentrations were determined with the bicinchoninic acid method (Pierce) using bovine serum albumin as standard. Samples containing concentrations were determined with the bicinchoninic method. Protein 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-repleted conditions using TRIzol (Invitrogen) 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 Molecular Biochemicals) using standard protocols (25, 27). Following the transfer, RNA was covalently bound to the membrane by cross-linking with 0.120 J/cm² UV light of a 254-nm wavelength. RNA was visualized by methylene blue staining (25), and RNA samples were normalized based on 16 S and 23 S rRNA band intensities. Internal fragments of the amiE and amiF genes were PCR-amplified with primers listed in Table I. 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 polynucleotide (Roche Molecular Biochemicals). Northern hybridization and stringency washes were performed at 68 °C, and bound probe was visualized with the DIG detection kit (Roche Molecular Biochemicals) and the chemiluminescent substrate CDP-Star (Amersham Biosciences) (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 the manipulation of DNA and transformation of E. coli (27) and H. pylori (26). Plasmid DNA was prepared using Qiagen spin columns (Qiagen). 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-labeled amiE and amiF promoter fragments were amplified with primer combinations Amid-PrF/Amid-PrR-DIG and Form-PrF/Form-PrR-DIG, respectively, 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 dithiothreitol, 300 μg/ml bovine serum albumin, 100 μM MnCl2, 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 200 V. The gel was then blotted onto a nylon membrane (Roche Molecular Biochemicals), and DIG-labeled DNA was visualized using the DIG detection kit (Roche Molecular Biochemicals) and the chemiluminescent substrate CDP-Star.

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 the induction of amidase expression upon supplementation of growth medium with the amide substrate (30, 31). Although orthologous of the corresponding amidase regulatory proteins are absent in H. pylori, the 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).

To determine whether amidase and formamidase activity were 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 \textit{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 the 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-repleted conditions (Fig. 1A). In contrast, formamidase activity was not changed in iron-restricted conditions when compared with iron-repleted conditions (Fig. 1B). Thus, we conclude that amidase and formamidase activity in \textit{H. pylori} 26695 is not substrate-inducible but that amidase activity is regulated by iron availability, whereas formamidase activity seems constitutive.

\textbf{Amidase Expression and Activity and Formamidase Activity Are Regulated by Fur—} The AmiE (HP0294) protein was previously identified as a protein of approximately 45 kDa with a \( p_I \) of 6.4 (33). A protein of similar molecular mass and \( p_I \) was identified when comparing two-dimensional protein profiles for the identification of Fur- and iron-regulated proteins of \textit{H. pylori} 26695 (Fig. 2). Wild-type cells expressed this protein when grown in iron-restricted conditions but not in iron-repleted conditions. This iron-repression was absent in the \textit{fur} mutant strain (Fig. 2), suggesting that iron regulation was mediated by Fur. Subsequent identification of the protein by mass spectrometry confirmed that this iron- and Fur-repressed protein was indeed AmiE (13, 14). Because the AmiF protein has not been identified on two-dimensional 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 \textit{H. pylori} 26695 and its isogenic \textit{fur} mutant grown in iron-restricted and iron-repleted 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-repleted conditions (\( p < 0.01 \)), whereas in the \textit{fur} mutant, activity was always high, independent of iron availability (Fig. 3A, \( p = 0.56 \)). Surprisingly, formamidase activity was also affected by the \textit{fur} mutation. Formamidase activity did not differ between cells grown in iron-restricted and iron-repleted conditions but differed significantly between the wild-type and \textit{fur} mutant cells (Fig. 3B, \( p < 0.01 \)). In wild-type cells, formamidase activity was low but present, whereas formamidase activity was increased almost 3-fold in the \textit{fur} mutant (Fig. 3B). These results were reproduced with a second independently constructed \textit{H. pylori} 26695 \textit{fur} mutant (data not shown), indicating that the increase in formamidase activity is not caused by a secondary mutation.

\textbf{Fur Mediates the 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 \textit{amiE} mRNA detected in the wild-type strain under iron-repleted conditions, but the transcription of a 1-kilobase mRNA was clearly apparent in \textit{fur} mutant (Fig. 4). However, the effect of the \textit{fur} mutation on formamidase activity is not mediated at the transcriptional level, because the small changes in the levels of \textit{amiF} mRNA observed on Northern hybridizations (Fig. 4) did not correlate with the changes in the enzyme activity observed (Fig. 4).
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 was indeed differentially regulated by Fur, we performed gel retardation assays using recombinant H. pylori Fur (29) and DIG-labeled amiE and amiF promoter regions. The addition of recombinant H. pylori Fur with the metal cofactor Mn²⁺ to the amiE promoter region shifted the 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 (data not shown). To check sequence specificity, we also used an internal fragment of the amiE gene whose mobility was not affected by Fur (data not shown). Finally, as predicted from the Northern hybridization experiments but despite the presence of Furbox-like sequence, the 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 although 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 reg-

**FIG. 3.** Effect of varying iron availability on the activity of amidase (A) and formamidase (B) activity in H. pylori 26695 wild-type and fur mutant strains. Enzyme activities were compared in lysates of cells grown in iron-repleted conditions (black bars) and iron-restricted (white bars) conditions, and their respective enzyme activities were determined. Graphs represent a minimum of five independent experiments, and error bars denote ± S.D. Statistical evaluation of the comparison of enzyme activities using the Mann-Whitney U test are given.

**FIG. 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-repleted (+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.

**FIG. 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 (32) are underlined. The distance of the putative Furboxes to the ribosome binding site 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.
ululatory 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 (1). 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 because of alkalinization of the medium to toxic levels at a 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. Although it is difficult to envisage high levels of amides being produced intracellularly in *H. pylori*, recent reports of possibly toxic or carcinogetic 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, because 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 the 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 although the urease reaction results in alkalinization of the environment (39), the amidase reaction is pH-neutral (13, 14). 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, because ammonia also plays an important role in nitrogen metabolism, the pH-neutral production of ammonia by both amidases may allow the 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 that are thought to deliver pro-inflammatory proteins to the epithelial cells but only in iron-repleted conditions (47). Combined, this would result in high urea release in iron-repleted 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, whereas the *fur* mutation only affects enzyme activity of AmiF but not *amiF* transcription (Figs. 4 and 5). The mechanism behind the increased formamidase activity in the *fur* mutant is currently unknown. We hypothesize that this increase may be the result of 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 the observed effect on formamidase activity is unlikely to 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 environment.
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