Presence of active aliphatic amidases in Helicobacter species able to colonize the stomach

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Presence of Active Aliphatic Amidases in Helicobacter Species Able To Colonize the Stomach

Stéphanie Bury-Moné,1 Stéphane Skouloubris,2 Catherine Dauga,3 Jean-Michel Thibeuge,1 Daiva Dailidienë,4 Douglas E. Berg,4 Agnès Labigne,1 and Hilde De Reus1*

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Ammonia production is of great importance for the gastric pathogen Helicobacter pylori as a nitrogen source, as a compound protecting against gastric acidity, and as a cytotoxic molecule. In addition to urease, H. pylori possesses two aliphatic amidases responsible for ammonia production: AmiE, a classical amidase, and AmiF, a new type of formamidase. Both enzymes are part of a regulatory network consisting of nitrogen metabolism enzymes, including urease and arginase. We examined the role of the H. pylori amidases in vivo by testing the gastric colonization of mice with H. pylori SS1 strains carrying mutations in amiE and/or amiF and in coinfection experiments with wild-type and double mutant strains. A new cassette conferring resistance to gentamicin was used in addition to the kanamycin cassette to construct the double mutation in strain SS1. Our data indicate that the amidases are not essential for colonization of mice. The search for amiE and amiF genes in H. pylori 53 strains from different geographic origins indicated the presence of both genes in all these genomes. We tested for the presence of the amiE and amiF genes and for amidase and formamidase activities in eleven Helicobacter species. Among the gastric species, H. acinonychis possessed both amiE and amiF, H. felis carried only amiF, and H. mustelae was devoid of amidases. H. muridarum, which can colonize both mouse intestine and stomach, was the only enterohepatic species to contain amiE. Phylogenetic trees based upon the sequences of H. pylori amiE and amiF genes and their respective homologs from other organisms as well as the amidase gene distribution among Helicobacter species are strongly suggestive of amidase acquisition by horizontal gene transfer. Since amidases are found only in Helicobacter species able to colonize the stomach, their acquisition might be related to selective pressure in this particular gastric environment.

Helicobacter pylori is a spiral-shaped, gram-negative bacterium that colonizes the gastric mucosa of humans (11, 36). It is the etiologic agent of chronic gastritis and peptic ulcers and is a risk factor for the development of gastric cancer (14). In H. pylori, nitrogen metabolism is strongly dependent on ammonia production, as illustrated by the absence of the GOGAT assimilation enzyme (glutamate synthase), which is characteristic of an organism in which ammonia is never limiting (9). One specific feature of H. pylori is the link between ammonia production and its capacity to resist strong acidity. Urease, which is very abundant in H. pylori, produces ammonia and is essential for the acid resistance of this bacterium (7, 13). Several studies have suggested that the large amounts of ammonia produced by H. pylori are also responsible for tissue damage during colonization. The long-term administration of ammonia induces mucosal atrophy in the stomachs of rats (51), and the ammonia generated by H. pylori accelerates cytokine-induced apoptosis in gastric epithelial cells (25). H. pylori also possesses other ammonia-producing enzymes, including deaminases, deamidases (both found in many bacteria), and, more unusually, two aliphatic amidases, AmiE and AmiF. Aliphatic amidases (EC 3.5.1.4) are enzymes that hydrolyze short-chain amides to produce ammonia and the corresponding organic acid. The finding of aliphatic amidases in H. pylori was unexpected, as these enzymes had been described only in bacteria that spend at least part of their life cycles in the environment (Pseudomonas aeruginosa and Rhodococcus erythropolis). In previous studies (42, 43), the two amidases found in H. pylori were identified and characterized. The first amidase, AmiE, is a typical aliphatic amidase, showing 75% sequence identity with its orthologs and presenting characteristic substrate specificity. In vitro, AmiE hydrolyzes acrylamide, acetamide, and propionamide very efficiently but is not active on formamide or urea (42). H. pylori is the first organism in which a second amidase has been found; this enzyme, designated AmiF, displays 34% sequence identity with AmiE proteins. AmiF displays unexpected substrate specificity, as it is active only on formamide, the shortest amide (42). AmiF is the first formamidase (EC 3.5.1.49) described to belong to the aliphatic amidase enzyme family. A conserved cysteine residue within the active site has been identified for both enzymes (42). Despite this knowledge of the amidases of H. pylori, the role of these enzymes in vivo and the nature and origin of the natural substrate of AmiE remain unclear. It is difficult to identify this substrate, as it can be generated either in the gastric juice or as a by-product of intracellular metabolism. Interestingly, we found that the production of AmiE and AmiF is dependent on the activity of other enzymes involved in nitrogen metabolism, urease and arginase, respectively (42). These data strongly suggest that the
amidases are involved in nitrogen metabolism in *H. pylori* and that their synthesis is regulated to maintain the intracellular nitrogen balance. AmiE is a highly produced protein. Antibodies specifically recognizing this protein, but not AmiF, are present in the sera of a large proportion of patients (21 out of 26 tested) (43; unpublished data). Immunoproteomic studies also confirmed the recognition of AmiE by patient sera. Serum samples from patients with gastric cancer were more frequently immunoreactive (22).

The aims of this study were (i) to improve our understanding of the role of amidases in *H. pylori* by using a mouse model and (ii) to examine the distribution of the amidases in the *Helicobacter* genus to allow us to determine whether there is a correlation between these enzymes and the habitat of the *Helicobacter* species tested (gastric, hepatic, or enteric). The distribution of the *amiE* and *amiF* genes in *H. pylori* and in the *Helicobacter* species led us to propose a model for the acquisition of these genes in relation to their colonization site.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *E. coli* strain MC1061 (5) was used as a host for the preparation of the plasmids used to transform *H. pylori*. *E. coli* strains were grown at 37°C on solid or liquid Luria-Bertani medium (35). Anti-*H. pylori* E. coli strain 6514 BURY-MONE was used as a host for the preparation of the plasmids used to transform *E. coli*. The strains used in this study are listed in Table 1. The strains were used at the following concentrations: kanamycin, 20 μg ml⁻¹; tetracycline, 10 μg ml⁻¹; gentamicin, 30 μg ml⁻¹; and carbenicillin, 100 μg ml⁻¹. The strains used in this study are listed in Table 1. *Helicobacter* strains were grown on blood agar base 2 plates (Oxoid) supplemented with 10% defibrinated horse blood and with an antibiotic and fungicide mix consisting of vancomycin (final concentration, 10 μg ml⁻¹), polynym B (2.5 IU liter⁻¹), trimethoprim (5 μg ml⁻¹), and fungizone (2.5 μg ml⁻¹). The plates were incubated for 24 to 48 h at 37°C in microaerobic conditions. For the selection of *S. suis* *H. pylori* transformants (four to five days of growth), kanamycin and gentamicin were added to the growth medium at concentrations of 20 μg ml⁻¹ and 5 μg ml⁻¹, respectively.

**Molecular techniques, PCR, sequencing, and Southern blotting.** Standard procedures were used for small-scale plasmid preparation, endonuclease digestion, ligation, and agarose gel electrophoresis and for the elution of DNA fragments from agarose gels (41). Midi or maxi QIAGEN columns were used for large-scale plasmid preparations. The QIAamp DNA extraction kit (QIAGEN) was used to extract chromosomal DNA from *Helicobacter* species. PCR was carried out according to the manufacturer’s recommendations by using the *Tag* DNA polymerase kit (Amersham). Sequencing was performed on an ABI 310 automated DNA sequencer (Applied Biosystems) as previously described (29). The chromosomal DNA extracted from the collection of *H. pylori* strains from different geographic origins was amplified by PCR with primers H46 and H58 for the *amiE* gene and primers H69 and H70 for the *amiF* gene (Table 2). The procedure comprised an initial denaturation step at 94°C (2') followed by 30 cycles of 30 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The absence of PCR cross-reaction on the cognate amide gene was verified with each pair of primers by using as a template *E. coli* plasmids in which the entire *H. pylori amiE* or *amiF* gene was cloned (pILL417 and pILL439b, respectively) (42). Southern blot experiments were performed by using a low-stringency hybridization technique with a mixture of both types of molecule; 5' corresponds to inosine.

**TABLE 1. Strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristic(s) or natural host</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC1061</td>
<td>Parental strain</td>
<td>16</td>
</tr>
<tr>
<td>26695</td>
<td>Parental strain</td>
<td>49</td>
</tr>
<tr>
<td>SS1</td>
<td>Parental strain</td>
<td>32</td>
</tr>
<tr>
<td>SS1-344a</td>
<td>SS1-amiEAamuac(3)-IV</td>
<td>This work</td>
</tr>
<tr>
<td>SS1-437b</td>
<td>SS1-amiFamuac-3</td>
<td>This work</td>
</tr>
<tr>
<td>SS1-444a-437</td>
<td>SS1-amiEAamuac(3)Wamauac-3</td>
<td>This work</td>
</tr>
<tr>
<td><em>H. pylori</em> (pluri-ethnic collection)</td>
<td>Human</td>
<td>D. E. Berg</td>
</tr>
<tr>
<td>H. acinonychis</td>
<td>Cheetah</td>
<td>12; via CIP*</td>
</tr>
<tr>
<td>H. bilis</td>
<td>Cat, dog</td>
<td>37; via R. Ferrero</td>
</tr>
<tr>
<td>H. canis</td>
<td>Ferret</td>
<td>19; via CIP</td>
</tr>
<tr>
<td>H. dekayi</td>
<td>Mouse, dog, human</td>
<td>10; via CIP</td>
</tr>
<tr>
<td>H. felis</td>
<td>Dog, human</td>
<td>52; via CIP</td>
</tr>
<tr>
<td>H. muridarum</td>
<td>Mouse, rat</td>
<td>20; via CIP</td>
</tr>
<tr>
<td>H. pylori</td>
<td>Mouse, rat</td>
<td>46; via CIP</td>
</tr>
<tr>
<td>H. pylori</td>
<td>Mouse</td>
<td>33; via CIP</td>
</tr>
<tr>
<td>ST1, ATCC 49282</td>
<td>33; via J. L. O'Rourke</td>
<td></td>
</tr>
<tr>
<td>ST2</td>
<td>33; via J. L. O'Rourke</td>
<td></td>
</tr>
<tr>
<td>ST3</td>
<td>33; via J. L. O'Rourke</td>
<td></td>
</tr>
<tr>
<td>ST5</td>
<td>18; via J. G. Fox</td>
<td></td>
</tr>
<tr>
<td>ST5</td>
<td>50; via CIP</td>
<td></td>
</tr>
</tbody>
</table>

* CIP, Collection of the Institut Pasteur.

**TABLE 2. Designations and nucleotide sequences of primers used for PCR amplification and plasmid construction**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer*</th>
<th>Oligonucleotide sequence (5' to 3')</th>
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<tbody>
<tr>
<td><em>amiE</em></td>
<td>H46(f)</td>
<td>CTTTATAACACCTTTGTACTTGT</td>
</tr>
<tr>
<td></td>
<td>H58(r)</td>
<td>CCCGCTACATAACACATAGTGGC</td>
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<tr>
<td></td>
<td>H49(r)</td>
<td>CAAGCCCTTATGGCCCATCAAC</td>
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<tr>
<td></td>
<td>H59(f)</td>
<td>CGGGATCCAGATAGGATAGTAGGGG</td>
</tr>
<tr>
<td></td>
<td>H37(r)</td>
<td>CCAAAT(C/T)TGGGG(G/A)TT(A/G)ATTCGAG</td>
</tr>
<tr>
<td></td>
<td>H59(f)</td>
<td>CCGGATCCATGGGTGATGCAGTGGG</td>
</tr>
<tr>
<td></td>
<td>H70(r)</td>
<td>CGaatttcGAATCATGCCGTCG</td>
</tr>
<tr>
<td></td>
<td>H011(r)</td>
<td>GGAAATTCTTCCTCCGTCGATCATCCGA</td>
</tr>
<tr>
<td></td>
<td>H90(r)</td>
<td>CAGCCTGACGATGATAC</td>
</tr>
<tr>
<td><em>amiF</em></td>
<td>H69(f)</td>
<td>CCGGATCCATGGGTGATGCAGTGGG</td>
</tr>
<tr>
<td></td>
<td>H70(r)</td>
<td>CGaatttcGAATCATGCCGTCG</td>
</tr>
<tr>
<td></td>
<td>H011(r)</td>
<td>GGAAATTCTTCCTCCGTCGATCATCCGA</td>
</tr>
<tr>
<td></td>
<td>H90(r)</td>
<td>CAGCCTGACGATGATGATAC</td>
</tr>
<tr>
<td><strong>Hsp RNA</strong></td>
<td>H276(f)</td>
<td>TATACGCGGTTATAGG</td>
</tr>
<tr>
<td></td>
<td>H276(r)</td>
<td>ATATCCGCTATATGATAGC</td>
</tr>
<tr>
<td></td>
<td>H37(r)</td>
<td>CCAAAT(C/T)TGGGG(G/A)TT(A/G)ATTCGAG</td>
</tr>
<tr>
<td></td>
<td>H59(f)</td>
<td>CCGGATCCATGGGTGATGCAGTGGG</td>
</tr>
<tr>
<td><strong>acc(3)-IV</strong></td>
<td>H121</td>
<td>GCGCTTGGGAGGCGCAAGTTG</td>
</tr>
<tr>
<td></td>
<td>H122</td>
<td>GACGTGGGGAGGCGCAAGTTG</td>
</tr>
<tr>
<td><strong>apki-A3</strong></td>
<td>H50</td>
<td>CGGGATATTCGTATTTTACC</td>
</tr>
<tr>
<td></td>
<td>H17</td>
<td>TTGACTACTCTGGGATCAAGCCTG</td>
</tr>
</tbody>
</table>

* f, forward primer; r, reverse primer.

* Lowercase letters indicate BamHI (H69) and EcoRI (H70) restriction sites. For primer H37, two residues at the same position indicate that the oligonucleotide preparation contained a mixture of both types of molecule; *t* corresponds to inosine.
ization protocol (hybridization at 42°C in the presence of 30% formamide) (41). Genomic DNA was digested overnight with the HindIII restriction enzyme. Gene-specific probes with chromosomal DNA from strain 26695 were obtained by PCR by using primer pair H59-H37 for amiE and primer pair H69-H89 for amiF; the probes for each gene were 522- and 654-bp long, respectively (Table 2). The probes were labeled with [α-32P]dCTP (3,000 Ci mmol−1) by using the Megaprime random labeling kit (Amersham Pharmacia Biotech). As a control, the same membranes were hybridized with a 16S rRNA gene probe (a 375-bp-long PCR product was generated with H276f, H676r, and H. pylori DNA) (Table 2) that recognized the corresponding genes in all the Helicobacter species.

Construction of plasmids and H. pylori mutants. As a prerequisite for the construction of a double mutation in strain SS1, we developed a new cassette suitable for insertional mutagenesis in this genetic background. Indeed, we were not able to select SS1 mutants with amiE interrupted by a cat cassette (encoding chloramphenicol acetyltransferase). Therefore, we used for the first time an apramycin-gentamicin resistance cassette originating from the pUC1813 vector (D. Mazel, unpublished data), a derivative of pUC1813 carrying the aac(3)-IV gene from the pWP7b R-plasmid (3). We verified the sequence of this gene and found that the originally published sequence contained more 5′ base pairs than our sequence (3). Partially corrected versions of the aac(3)-IV gene confers resistance to gentamicin, and the aphA-3 gene confers resistance to kanamycin. The chromosomal organization of the resulting mutant strains is shown with the adjacent genes. We used specific primers to verify that the genes downstream and upstream of amiE and amiF in strain SS1 were identical to those of the sequenced strain 26695. The small arrowheads indicate the primers used in this study.

The wild-type SS1 strain and the single mutants (SS1-437b and SS1-444a) had the wild-type SS1 strain and the single mutants (SS1-437b and SS1-444a) had the same gastric tissues were used for the quantitative culture of H. pylori on blood agar plates in the presence of bacitracin (200 μg ml−1) and nalidixic acid (10 μg ml−1) as described before (17): (i) without antibiotics for the mice infected with the SS1 parental strain, (ii) with 20 μg of kanamycin ml−1 for mice infected with SS1-437b, (iii) with 5 μg of gentamicin ml−1 for mice infected with SS1-444a, (iv) with kanamycin and gentamicin for mice infected with SS1-444a-437b, and (v) on plates without antibiotics plus on plates with both kanamycin and gentamicin for the mixed infections. We confirmed that with the mutant strains the presence of one or two antibiotics did not affect the number of CFU. The wild-type SS1 strain and the single mutants (SS1-437b and SS1-444a) tested for the presence of H. pylori with a direct urease test performed on half the stomach.

Mouse model for colonization. Wild-type or mutant strains of H. pylori were harvested after 24 h of growth on blood agar plates and suspended in peptone broth. The bacterial concentrations were adjusted to 106 bacteria ml−1 or 107 bacteria ml−1, depending on the experiment; the CFU were subsequently counted precisely. Aliquots (100 μl) of bacteria were administered orogastrically to 10 female NMRI mice (four weeks old; provided by Iffa-Credo) as described previously (17). In each experiment, six mice were inoculated with peptone broth as a negative control. Mice were killed 4 weeks after inoculation. We tested for the presence of H. pylori with a direct urease test performed on half the stomach. The remaining gastric tissues were used for the quantitative culture of H. pylori on blood agar plates in the presence of bacitracin (200 μg ml−1) and nalidixic acid (10 μg ml−1) as described before (17): (i) without antibiotics for the mice infected with the SS1 parental strain, (ii) with 20 μg of kanamycin ml−1 for mice infected with SS1-437b, (iii) with 5 μg of gentamicin ml−1 for mice infected with SS1-444a, (iv) with kanamycin and gentamicin for mice infected with SS1-444a-437b, and (v) on plates without antibiotics plus on plates with both kanamycin and gentamicin for the mixed infections. We confirmed that with the mutant strains the presence of one or two antibiotics did not affect the number of CFU. The wild-type SS1 strain and the single mutants (SS1-437b and SS1-444a) had the wild-type SS1 strain and the single mutants (SS1-437b and SS1-444a) had the wild-type SS1 strain and the single mutants (SS1-437b and SS1-444a) had the wild-type SS1 strain and the single mutants (SS1-437b and SS1-444a) had the wild-type SS1 strain and the single mutants (SS1-437b and SS1-444a) had
undergone 21 in vitro subcultures since recovery from the mouse stomach, whereas the double mutant strain (SS1-444a-437b) had undergone 25. This is far below the number of in vitro cultures at which the SS1 strain starts to lose its ability to colonize mice (about 75 passages). R. Ferrero, personal communication. To examine the growth of the mutant strains (single or double mutants) in liquid medium to ensure that their doubling time was identical to that of the parental SS1 strain.

Measurement of urease, amidase and formamidase activities. Cell extracts prepared as previously described (43) were used to measure urease, amidase, and formamidase activities. Samples (10 to 50 μl) were added to 200 μl of a urea or amid substrate solution (acrylamide, acetamide, or formamide) at a final concentration of 100 mM in 100 mM phosphate buffer, pH 7.4, and 10 mM EDTA. The reaction mixture was incubated at room temperature for 30 min; then 400 μl of phenol-nitroprusside and 400 μl of alkaline hypochlorite were added, and the samples were incubated for 6 min at 50°C. Absorbance was then read at 625 nm. The amount of ammonia released was determined from a standard curve. One unit (U) of amidase or formamidase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of amide (corresponding to the formation of 1 μmol of ammonia) per min per mg of total protein. One unit of urease activity was defined as the amount of enzyme required to hydrolyze 1 μmol of urea (producing 2 μmol of ammonia) per min per mg of total protein. Protein concentration was determined with a commercial version of the Bradford assay (Sigma Chemicals) by using bovine serum albumin as a standard.

Phylogenetic analysis of the amiE and amiF genes. Sequences of amiE and amiF were retrieved from the EMBL (Cambridge, United Kingdom) and Gen-Bank (Bethesda, Md.) databases. The amiE sequences of Bacteroides fragilis and Desulfovibrio vulgaris were obtained from the respective unfinished genome sequences of the Institute of Genome Research. The amiE and amiF sequences data sets were treated separately. Sequences were first aligned by using Clustal W (24). The initial alignments were further refined by eye by using the alignment editor of the PAUP* version 4.0 software package (47). Likelihood scores were calculated for 56 different models of evolution by using PAUP*. The likelihood scores were compared by using the hierarchical likelihood ratio test approach through Modeltest version 3.0 (38). The general time reversible model of substitution (39) was selected as the model that best fit both gene data sets. Gamma shape parameters, substitution rate matrices, and nucleotide frequencies were also estimated for each gene by using Modeltest. The improved version of the Neighbor Joining algorithm, BIONJ (21), and the maximum likelihood method were applied under these models and settings to build phylogenetic trees. Maximum parsimony trees were built by heuristic search with a starting tree based on 10 random stepwise additions and using the tree bisection-reconnection branch-swapping option. Statistical significance was evaluated by bootstrap analysis (15) with 100 repeats of bootstrap samplings.

RESULTS

Use of a new apramycin-gentamicin resistance cassette to generate amidase gene mutations in the SS1 strain. The mouse-adapted H. pylori SS1 strain is widely used for its capacity to colonize mice efficiently and because it is genetically transformable (32, 44). To construct an SS1 mutant strain carrying insertional mutations in both amidase genes, we used a new cassette comprising a gentamicin-apramycin resistance gene, designated aac(3)-IV and originating from the pWP7b R-plasmid (3). This cassette was constructed and validated in different H. pylori strains, including SS1, N6, 26695 (Table 1), J99 (2), X47-2AL (27), and HAS-141 (26). The aac(3)-IV gene was inserted into the amiE gene in both orientations (pILL444a and pILL444b). After its transformation into H. pylori strain SS1 and its selection on gentamicin, we found that allelic exchange occurred only with pILL444a, i.e., when aac(3)-IV was in the same orientation as amiE (Fig. 1). The resulting strain was designated SS1-444a (Fig. 1). This suggests that the aac(3)-IV promoter was not active in H. pylori and that it was expressed only under the control of the amiE promoter in strain SS1-444a.

The same strategy, inserting pILL437b carrying the aphA3 gene into the amiF gene, was used to construct strain SS1-437b (Fig. 1). An SS1 derivative deficient in both amidase genes was constructed by introducing the amiF1aphA3-3 mutation into the SS1-444a [amiE1aac(3)-IV] strain. This double-mutant SS1 derivative carrying amiE1aac(3)-IV and amiF1aphA3 was designated SS1-444a-437b (Table 1). We ensured that the single mutants lacked AmiE or AmiF activities and that the double mutant lacked AmiE and AmiF activities.

Colonization of the mouse model with amidase-deficient mutants. We used several different experimental approaches to study the impact of amidase and formamidase activities on the efficiency of mouse colonization by the SS1 strain (Fig. 2). In the first protocol, the inoculation dose was 1 × 10^6 to 7 × 10^9 bacteria per mouse. The infecting strains consisted of the single-mutant strains deficient in amiE (SS1-444a) or in amiF (SS1-437b), the double-mutant strain (SS1-444a-437b), and the parental wild-type SS1 strain (positive control). Four weeks after infection, we assessed the bacterial load in the mouse stomach. In these conditions, colonization with the mutant strains was as effective as that with the parental strain, with mean gastric colonization loads of 5 × 10^8 to 3 × 10^9 bacteria per g of gastric tissue (Fig. 2A). Similar results were obtained with amidase-deficient mutants of another mouse-adapted strain, X47-2AL (27) (data not shown).

In a second set of experiments, the inoculation dose was between 4 × 10^7 and 8 × 10^7 bacteria per mouse. We compared the outcomes of the infection with the wild-type strain and with the amidase double mutant strain, and we also performed coinfections to examine whether the double mutant strain could be out-competed for colonization by the wild-type strain. We first tested a mixed infection with 50% wild-type strain (4 × 10^7 bacteria/mouse) and 50% double mutant strain (4 × 10^7 bacteria/mouse). This test allowed us to determine which of the two strains colonized the mouse stomach and to what extent, based on the kanamycin and gentamicin resistance of the double mutant. In this experiment, we found that the mean colonization load for the double mutant alone was higher than that for the wild-type strain alone (9 × 10^7 and 4 × 10^7 bacteria per g of gastric tissue, respectively) (Fig. 2B). Nine of the 10 mice infected with the mixed inoculum were colonized as follows (Fig. 2, table): (i) four mice carried almost exclusively the wild-type strain; (ii) three mice were infected only with the double mutant strain; and (iii) two mice were colonized with a mixed population. The unexpectedly low proportion of mice presenting mixed colonization will be discussed later.

In the last experimental approach (Fig. 2C), the inoculation dose was slightly lower (10^7 bacteria per mouse). This was probably a limiting dose, as for each infecting strain 3 or 4 of the 10 mice were not infected. The mean colonization loads of the wild type and the double mutant were 4.5 × 10^4 and 1.5 × 10^4 bacteria per g of gastric tissue, respectively. In the colonization competition test, we inoculated mice with a mixture of 10% wild-type strain (10^6 bacteria/mouse) and 90% double mutant strain (9 × 10^6 bacteria/mouse). Six mice were infected. The double mutant strain was the unique colonizer in four mice, and the wild-type strain was the only colonizer in one case. The last mouse was colonized with a mixture of both strains (Fig. 2, table). The competition index was calculated for the coinfection experiments as previously described (40). This value, which takes into consideration the outcome of the bac-
terial load for each of the two inoculated strains, showed that in the mouse model the double mutant strain was not at a disadvantage for colonization.

Distribution of the amiE and amiF genes in *H. pylori* isolates. Given the great variability and plasticity of the *H. pylori* genome (1), we investigated whether the amiE and amiF genes were always present together in this species. We tested 53 *H. pylori* strains from various geographic origins by PCR with amiE-specific primers (H46-H58) (Table 2) and with amiF-specific primers (H69-H70) (Table 2). The size of the corresponding PCR products was 307 bp for amiE and 514 bp for amiF. The strains included 20 strains from Bordeaux in France (which already tested positive in previous studies; see references 42 and 43), 5 from Spain, 5 from Lithuania, 5 from Hong Kong, 5 from India, 5 from South Africa, 4 from Peru, and 4 from Alaska. PCR products of the expected size were obtained in every case, indicating that all strains examined contained both the amiE and amiF genes.

Construction of phylogenetic trees for the amiE and amiF genes. Helicobacter is the only bacterium from the gastrointestinal and urinary tracts known to possess an aliphatic amidase and a new type of formamidase. This prompted us to investigate the evolutionary origin of these enzymes by establishing phylogenetic trees for these two genes.

As a prerequisite to this analysis, the general time reversible model of substitution was applied to our data. The parameters estimated under this model showed slight differences between base frequencies and between substitution rates among sites of the two genes. Nucleotide frequencies were found to be equal for amiF and heterogeneous for amiE (for A, 0.2609; for C,

![Graph showing bacterial load for each of the two inoculated strains.](image)

**FIG. 2.** Assessment of mouse stomach colonization after inoculation with *H. pylori* SS1, amidase-negative mutant strains, or a mixture of wild-type and mutant strains. SS1-444a corresponds to the amiE-deficient mutant, SS1-437b corresponds to the amiF mutant, and SS1-444a-437b corresponds to the double mutant strain deficient in both amidases. Panels A, B, and C correspond to three independent experiments. The strains used to inoculate the mice are indicated below the panels. The detection limit of the assay is indicated by the dashed horizontal line. PB corresponds to five control animals inoculated with sterile peptone broth. Each point (indicated by an open diamond) corresponds to the gastric colonization load for one mouse, and the horizontal bars represent the geometric mean for each group of mice. The table presents the results of the two coinfection experiments. wt indicates the wild-type strain, and mt (amiE/amiF) indicates the double mutant. The values in the table correspond to the number of mice presenting a given stomach colonization profile versus the total number of mice tested for the coinfection experiment of panel B or C. For instance, in the panel B coinfection experiment, 4 out of the 10 inoculated mice were infected solely with a wild-type strain.
Both genes showed variable substitution rates over sites, but the extent of rate variation was more apparent for amIE. The alpha shape parameters (used to determine the shape of the gamma distributions of these rates) were 1.0695 and 0.4315 for amIE and amIF, respectively. Thus, the parameters estimated for the two samples of sequences revealed that there were differences in the evolutionary constraints between amIE and amIF.

The phylogenetic trees based on the amIE and amIF genes obtained with distance methods are shown in Fig. 3a and b, respectively. The 1,020 and 1,033 unambiguously aligned positions obtained with distance methods are shown in Fig. 3a and b, respectively. The percentages are the significant bootstrap values of 100 calculated trees.

The phylogenetic trees based on the amIE and amIF genes were rooted by genes like gene, and H. muridarum was found to contain an amIE-like gene. No hybridization product was detected for the other species examined.

To further investigate the distribution of the amidases in the Helicobacter genus, we measured enzymatic activities on crude extracts for these Helicobacter species and for the two H. pylori strains (N6 and 26695) used as controls. For every strain, three different enzymatic activities were tested by measuring the ammonia produced in the presence of (i) urea for urease activity, (ii) formamide for AmiF-like formamidase activity, and (iii) acrylamide and acetamide for AmiE-like amidase activity (Table 3). As previously reported (45), urease activity was detected for all species except H. pametensis, H. cinaedi, H. canis, and H. fennelliae. Strong formamidase activity was detected for H. acinonychis and H. felis, whereas weak formamidase activity was detected for H. mustelae and activity close to the limit of detection was found for H. muridarum. Low formamidase activities could result from urease cross-reactivity on formamide, as shown previously with an H. pylori amIE mutant (42). This finding is in contrast with that for AmiE activity, which was below the limit of detection (<0.01 U) in an H. pylori amIE mutant.

Amidase activities with acrylamide and acetamide were found for H. acinonychis and H. muridarum in addition to the two H. pylori strains. H. muridarum was the only enterohepatic
strain to display AmiE-like amidase activity. This unexpected result was confirmed by the measurement of the same AmiE-like amidase activities in three other *H. muridarum* isolates (ST2, -3, and -5) (Table 1). In addition, when positive, the AmiE amidase activity was always detected with both acrylamide and acetamide, suggesting a significant conservation of this enzyme.

**DISCUSSION**

*H. pylori* presents the original characteristic of possessing two amidases belonging to the aliphatic amidase family even though these enzymes are usually found only in bacteria from the environment. The hydrolysis of short-chain amides by the amidases provides the cell with ammonia. Ammonia is a central compound in *H. pylori*, as it is a major nitrogen source, contributes to the exceptional capacity of this organism to resist gastric acidity, and has a cytotoxic potential. We previously characterized the general aliphatic amidase, AmiE, and the AmiF formamidase (42, 43) of *H. pylori* and identified their in vitro substrates. The amidases are part of the nitrogen metabolic pathway of *H. pylori*; however, the origin of their natural substrates and the nature of the in vivo AmiE substrate remain unknown. We showed that the expression of the two amidases is dependent on the activity of two enzymes, urease and arginase, known to play a key role in nitrogen metabolism and colonization by this organism (42, 43). We proposed the existence of a common transcriptional regulatory network designed to maintain the intracellular nitrogen balance, to avoid toxic intracellular accumulation of ammonium, and possibly to respond to acid stress (9, 42). As *H. pylori*, with its unique gastric habitat, is still the only nonenvironmental bacteria to possess these enzymes, we hypothesized that these functions might have been selected through evolution because they confer a certain advantage to this bacterium for the colonization of its particular niche. Our in vivo competition experiments with

FIG. 4. Distribution within the *Helicobacter* phylogenetic tree of amidase activities and the *amiE* or *amiF* genes. The 11 *Helicobacter* species tested are shown in bold. A hatched line separates the gastric species (top) from the enterobacterial species (bottom). The tree (adapted from the work of Solnick and Schauer [45]) contains a total of 28 *Helicobacter* species and is based on 16S rRNA sequences. The scale bar represents a 1% difference in nucleotide sequence, as determined by measuring the lengths of the horizontal lines connecting two species. The results of our study are indicated in shaded boxes; the black borders indicate that amidase or formamidase was found. The following abbreviations are used: Ure, AmiE, and AmiF for urease activity, AmiE-like activity, and AmiF-like enzyme activities, respectively, and *amiE* and *amiF* for the amidase and formamidase gene, respectively.
the mouse did not detect a selective advantage for the strains possessing the amidases, but the mouse model might be badly suited to testing for subtle metabolic selective advantages. The potential influence of the amidase-produced ammonium on the degree of local gastric inflammation of mice still remains to be investigated.

Surprisingly, in our coinfection experiments, we observed that most of the mice were colonized with a single population corresponding to either of the two inoculated strains. Our interpretation is that only a very small subpopulation of the corresponding to either of the two inoculated strains. Our finding was that most of the mice were colonized with a single population that might play a nonessential metabolic role in the colonization of mice still remains to be investigated.

Results for the presence of amidase or formamidase activities and the presence of the corresponding genes. Among the four gastric Helicobacter species, the H. felis-like formamidase and AmiF activities and the corresponding AmiF-like formamidase. Examples of gastric Helicobacter species are H. pylori, H. felis, H. acinonychis, and H. mustelae, which colonize the stomachs of humans, cats, cheetahs, and ferrets, respectively. The tropism of the enterohepatic Helicobacter species is more diverse, and these species have been identified in the intestinal tracts and/or the livers of humans, other mammals, and birds.

In our analysis, there was a good correlation between the detection of amidase or formamidase activities and the presence of the corresponding genes. Among the four gastric Helicobacter species, tested, we observed that H. pylori and a closely related species, H. acinonychis, possessed both the AmiE amidase and the AmiF formamidase (Table 3). H. felis possessed only an AmiE-like amidase and formamidase. H. mustelae was devoid of both AmiE and AmiF activities and the corresponding genes. Very weak formamidase activity was measured, possibly due to a side activity of another enzyme such as urease, which is highly active in this organism. Interestingly, although H. mustelae is a ferret gastric pathogen, it is phylogenetically closer to the enterohepatic species than to the gastric species (45). H. muridarum was the only enterohepatic species tested found to display general amidase activity and to possess the corresponding AmiE gene. This unexpected result is particularly interesting, given that H. muridarum is the only enterohepatic Helicobacter species known to colonize the mouse stomach in addition to its primary site in the intestine (45). In some cases, inflammatory lesions such as gastritis were found to be associated with stomach colonization by H. muridarum in mice.

These results indicate that amidases are found only in Helicobacter strains that are able to colonize the stomach, al-
though they cannot be considered to be a specific marker for
gastric Helicobacter. This type of association is what is
expected from a metabolic function; having an amidase might
enhance fitness in a particular environment and thus help a pathogen
come more successful. However, having an amidase is not
essential for the colonization of this niche, as suggested by our
in vivo experiments and by the absence of amidase in H. mus-
telae. Amidases are not found in urease-negative Helicobacter
species, supporting the notion that the activities of urease,
amidase, and formamidase are coordinated.

To further investigate the link between the distribution
of the amidases among the Helicobacter species and the tropism
of these strains, we performed a phylogenetic analysis of these
unusual enzymes to get insight into their origin and evolution-
ary history. The degree of identity between AmiE from H.
pylori and its homologs from phylogenetically distant organ-
isms is abnormally high: 74% for the homolog from P. aerugi-
nosa and 74.3 and 76% for those from R. erythropolis and G.
stearothermophilus, respectively (42, 43). The same observation
is true for AmiF, for which two highly conserved homologs
were recently found in the unfinished sequences of two environ-
tmental bacteria, B. anthracis (75.5% identity) and D. vulgaris
(74% identity). These high identity levels are parti-
cularly striking in comparison to the homologies between
H. pylori housekeeping proteins and their homologs. For ex-
ample, the MutY and GlmM proteins share 32 and 40% iden-
tity with their respective homologs from P. aeruginosa. Phy-
genetic analyses indicated that the strongest evolutionary
affinities of H. pylori amiE and amiF were with genes from
low-G+C-content, gram-positive bacteria (Fig. 3). The dis-
crepancy between the close relationship between the amidase
genes from H. pylori and Bacillus spp. and the large evolu-
tionary distance between the corresponding genomes is strongly
suggestive of lateral gene transfer. This hypothesis is supported
by the observation that the H. pylori amidase genes have atyp-
ical features in their own genomic context. The overall GC
content of H. pylori is 39%, whereas the GC contents of amiE
and amiF are 45 and 46.6%, respectively. These arguments,
in agreement with the criteria developed by Lawrence and
Ochman (30), give clues to the origin and direction of the lateral
gene transfer. Ancestors of H. pylori probably acquired the
amiE and amiF genes from the low-G+C-content, gram-
positive bacterial lineage. Our phylogenetic data and the per-
centage of identity between each AmiE protein and each AmiF
protein, around 34% whatever the organism, make it hard to
distinguish between (i) functional convergence of these two
enzymes and (ii) enzymes acquired by lateral gene transfer of
two deep or hidden paralogs generated by very ancient gene
duplication (maybe in a gram-positive organism). We favor this
last, more complex hypothesis, although hidden paralogs are,
by definition, difficult to identity (8).

When considering the presence of active amidases within the
Helicobacter phylogenetic tree (Fig. 4), we observed that amiE
and amiF are not systematically found together and are not
uniformly distributed. The most likely evolutionary scenario is
therefore the acquisition of alien amidase genes by different
transfer events after the speciation of Helicobacter (Fig. 4). As
both amiE and amiF are present together in two closely related
species, H. pylori and H. acinonychis, gene transfer events could
have first occurred in this phylogenetic branch. The minimal

evolutionary scheme implies that an additional lateral gene
transfer event took place, accounting for the presence of an
amiE gene in H. muridarum. This model is supported by the
absence of amidases in the closely related species H. hepaticus.
At least two hypotheses can be formulated: (i) that the H.
muridarum amiE gene was acquired from a non-Helicobacter
organism and, because of this new function, it became able to
colonize the stomach of mice more efficiently and (ii) that H.
muridarum acquired an amiE gene by DNA exchange with a
amidase-carrying gastric Helicobacter encountered during an
episode of stomach colonization.

In conclusion, our distribution approach and phylogenetic
analysis revealed original properties of the Helicobacter ami-
dase genes. Aliphatic amidases are found only in Helicobacter
species able to colonize the stomach. In addition, the amidase
genes of H. pylori were obtained by horizontal transfer, possi-
ble from a low-G+C-content, gram-positive organism. We hy-
pothesize that the acquisition of these amidase genes might
give the strain a metabolic advantage when colonizing an un-
usual ecological niche such as the stomach. It is noteworthy
that H. pylori has the particularity of possessing an arginase,
an enzyme-producing urea with a metabolic link to the amidases,
for which the closest homolog is from Bacillus subtilis (34).
It might not be a coincidence that B. subtilis is a low-G+C-
content, gram-positive organism like the postulated donor or-
ganism of the amidases. Finally, we believe that using this type
of genomic distribution approach to study the role of metabolic
proteins will provide new insights into the natural history and
adaptive potential of recently discovered pathogens such as
H. pylori.

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