Helicobacter pylori rocF Is Required for Arginase Activity and Acid Protection In Vitro but Is Not Essential for Colonization of Mice or for Urease Activity

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Arginase of the Helicobacter pylori urea cycle hydrolyzes 1-arginine to 1-ornithine and urea. H. pylori urease hydrolyzes urea to carbon dioxide and ammonium, which neutralizes acid. Both enzymes are involved in H. pylori nitrogen metabolism. The roles of arginase in the physiology of H. pylori were investigated in vitro and in vivo, since arginase in H. pylori is metabolically upstream of urease and urease is known to be required for colonization of animal models by the bacterium. The H. pylori gene hp1399, which is orthologous to the Bacillus subtilis rocF gene encoding arginase, was cloned, and isogenic allelic exchange mutants of three H. pylori strains were made by using two different constructs: 236-2 and rocF::aphA3. In contrast to wild-type (WT) strains, all rocF mutants were devoid of arginase activity and had diminished serine dehydratase activity, an enzyme activity which generates ammonium. Compared with WT strain 26695 of H. pylori, the rocF::aphA3 mutant was ~1,000-fold more sensitive to acid exposure. The acid sensitivity of the rocF::aphA3 mutant was not reversed by the addition of l-arginine, in contrast to the WT, and yielded a ~10,000-fold difference in viability. Urease activity was similar in both strains and both survived acid exposure equally well when exogenous urea was added, indicating that rocF is not required for urease activity in vitro. Finally, H. pylori mouse-adapted strain SS1 and the 236-2 rocF isogenic mutant colonized mice equally well: 8 of 9 versus 9 of 11 mice, respectively. However, the rocF::aphA3 mutant of strain SS1 had moderately reduced colonization (4 of 10 mice). The geometric mean levels of H. pylori recovered from these mice (in log10 CFU) were 6.1, 5.5, and 4.1, respectively. Thus, H. pylori rocF is required for arginase activity and is crucial for acid protection in vitro but is not essential for in vivo colonization of mice or for urease activity.

Helicobacter pylori causes gastritis (2, 14, 35), is strongly associated with the development of peptic ulcers (3, 16), and constitutes a risk factor for gastric adenocarcinoma (9, 27). Although the mechanisms behind the development of these diseases are not well understood, urease, which catalyzes the hydrolysis of urea to carbon dioxide and ammonium, is clearly central to the pathogenesis of H. pylori infection since urease is absolutely essential for colonization of a variety of animal models (4, 5; for a review, see references 19, 24, and 25).

In situ studies have shown that H. pylori synthesizes urea and ornithine from the catabolism of arginine (23). This reaction is catalyzed by arginase, an enzyme of the H. pylori urea cycle (21). Thus, in the metabolism of nitrogenous end products in H. pylori, arginase activity is upstream of urease (21). H. pylori may also obtain some of its urea requirements through arginase activity of the host, since some mammalian cells have a complete urea cycle (21, 38). H. pylori does not have the enzymes for arginine biosynthesis and is therefore dependent on host-derived arginine (1, 20, 21, 26, 29, 34). It is thus possible that urease, arginine, arginase, and the other enzymes of the urea cycle play a fundamental role in the release and assimilation of ammonium, thereby contributing to maintaining the nitrogen balance in H. pylori (21, 23). Indeed, most arginases play a crucial biological role in regulating cytosolic arginine and ornithine levels, which are required for numerous metabolic processes, such as protein synthesis and production of polyamines and nitric oxide (1a).

H. pylori is an acid-sensitive organism but is protected from acid (pH <4.0) by the ammonia released from urea hydrolysis due to urease activity (3a, 10, 15, 31, 33). Thus, H. pylori exposed to acid in vitro survive in the presence of urea; survival in the presence of other metabolites has not been reported. H. pylori is also able to generate ammonium via the actions of other enzymes, including an aliphatic amidase (32), and various amino acid deaminases, such as asparaginase, aspartase, glutaminase, and serine dehydratase (20). The latter four enzymes provide the bacterium with intracellular ammonium derived from the catabolism of the respective amino acids. It is currently unclear what role, if any, these ammonium-generating enzymes play in the protection of H. pylori from acid exposure.

Partially purified H. pylori arginase is a homo-oligomer (100 to 300 kDa) consisting of ~37-kDa subunits, each of which is predicted to contain two metal ions in the active site (23). H. pylori arginase is unique among the arginase enzyme family in that it has highest activity for cobalt rather than manganese, and the enzyme is associated with the cell wall fraction rather than the cytosol (23). H. pylori arginase has very high specificity
arginase and does not recognize the structurally similar compound agmatine. Previously, it was hypothesized that an open reading frame, hp1399, of *H. pylori* 26695 encoded the arginase since the deduced amino acid sequence of this protein showed 20 to 27% identity with other bacterial arginases, with greatest homology (27.1%) to *Bacillus subtilis* RocF (1, 8, 23, 34). In accordance with the *B. subtilis* gene encoding arginase, the putative arginase gene hp1399 of *H. pylori* was referred to as rocF (1, 34). However, there are no reports confirming that hp1399 actually encodes the *H. pylori* arginase, and it is not known whether *H. pylori* rocF is required either in vitro for growth or, given its close metabolic relationship with urea, in vivo for the colonization of mice.

To establish that rocF encodes arginase, the gene was cloned and rocF isogenic mutants of *H. pylori* were constructed by allelic exchange. To provide unambiguous functional data on *H. pylori* RocF, in vitro and in vivo studies were performed by using rocF mutants in which the rocF gene was either inactivated by gene disruption alone or, alternatively, by both gene disruption and deletion. The resulting rocF mutants and corresponding wild-type (WT) strains of *H. pylori* were tested for arginase activity by employing proton nuclear magnetic resonance (1H-NMR) spectroscopy. Since arginase may be metabolically coupled with other ammonium-generating enzymes, including urease, the WT and rocF mutants of *H. pylori* were also tested for urease and deaminase activities. Finally, the effects of rocF gene inactivation on the survival of *H. pylori* to acid exposure in vitro and on gastric colonization in the mouse model (7, 13) were investigated. The mouse model for *H. pylori* mimics the human disease in inflammation, in colonization location (i.e., antrum of the stomach), and in the degree of colonization.

**MATERIALS AND METHODS**

**Bacterial strains, growth conditions, primers, and plasmids.** *H. pylori* strains 26695, N6, and SS1 were grown on brucella agar with 10% (vol/vol) sheep defibrinated blood or on blood agar base number 1 or 2 with 10% (vol/vol) horse blood in a microaerobic environment for 2 to 3 days by using the CampyPak Plus system (Becton Dickinson). Kanamycin-resistant DNA plasmids were grown to medium as appropriate. *Escherichia coli* strains were grown on Luria (L) agar and in L broth plus appropriate antibiotics (ampicillin, 100 μg/ml; kanamycin, 50 μg/ml). For growth of *H. pylori* in broth, bacteria were inoculated to a starting *A*₀ of 0.05 in 100 ml of Mueller-Hinton broth with 3% heat-inactivated fetal bovine serum (Sigma Cell Culture, St. Louis, Mo.) and grown in a 250-ml flask under microaerobic conditions with aeration (200 rpm) for 2 days. The oligonucleotide primers, plasmids, and bacterial strains are listed in Table 1.

**Chemicals.** L-Arginine, L-asparagine, L-aspartate, L-arginine, L-glutamine, L-serine, and L-arginine, and urea were obtained from Sigma. All reagents were of the highest purity available. Sigma hypochlorite urease assay as described previously (37), with the modifications described earlier (18).

**PCR sequence analysis of each rocF mutant, the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham) was used. Chromosomal DNA from each strain of *H. pylori* was prepared by 2 μl of DNA from each strain of *H. pylori* was prepared by PCR amplification of DNA products corresponding to sequences both upstream and downstream of the kanamycin insertions sites in *H. pylori* rocF. PCR products were treated with 10 U of exonuclease I and 2 U of Klenow DNA polymerase (Quick-Quick, Qiagen, Inc., Valencia, Calif.). Plasmid DNA was isolated by the alkaline lysis method (30) or by column isolation (30) or by the Qiamp technique (Qiagen, Inc., Valencia, Calif.), according to the manufacturer’s instructions. Labeled DNA probe was generated by PCR amplification of DNA products corresponding to rocF sequences both upstream and downstream of the kanamycin cassette sites in *H. pylori* rocF. PCR products were purified on Qia-Quick (Qiagen) and were sequenced by using plasmid DNA and specific primers corresponding to the sequences flanking the kanamycin cassette sites. Sequencing reaction conditions were: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The sequencing reactions were carried out using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The sequence data was obtained by use of the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequencing gels were dried under vacuum and exposed to a phosphorimager system (Alpha Innotech, San Leandro, Calif.). The sequence data was obtained by use of the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequencing gels were dried under vacuum and exposed to a phosphorimager system (Alpha Innotech, San Leandro, Calif.).

**Urease extract preparations, protein determinations, and quantitative determination of urease activity by the phenol-hypochlorite method.** *H. pylori* cell samples were prepared as described previously (18). Protein concentrations were determined by the Bicinchoninic Acid assay method (Pierce Chemical Company, Rockford, Ill.), according to the manufacturer’s instructions. Urease activity extracts of *H. pylori* was determined by measuring the amount of ammonium release from urea in the phenol-hypochlorite urease assay as described previously (37), with the modifications described earlier (18). Data are presented as urease specific activity (nanomoles of ammonium per minute per milligram of protein). Statistical analysis of the data was done by use of the Alternative Welch’s t test with InStat 2.03 software (GraphPad Software, San Diego, Calif.).

**Preparation of cells and lysates for NMR spectroscopy.** *H. pylori* cell suspensions were prepared by harvesting cells in sterile NaCl (150 mM) centrifuging them at 17 000 × *g* (6°C, 8 min). The supernatant was discarded, and the pellet was collected and resuspended in 150 mM NaCl. The procedure was repeated three times. After the final wash, packed cells were resuspended to a concentration of 10^9 cells/ml in sterile 150 mM NaCl. Lysates were prepared by resuspending packed cells in sterile NaCl (150 mM) and suspending by two freeze-thaw cycles in liquid nitrogen.

**Quantitative determination of arginase, aspartase, asparaginase, glutamate, and serine dehydrogenase activities of *H. pylori* rocF mutants.** For the NMR measurements, cells suspended in a phosphate (20 mM, pH 7.0)-NaCl (115 mM)-KCl (15 mM) buffer were placed into 5-mm tubes (Wil-
products were observed by NMR spectroscopy. 1H-NMR free induction decays (FID) were recorded at 37°C. Substrates (L-glutamine (80 mM), L-serine (80 mM), L-arginine (100 mM), L-asparagine (80 mM), L-aspartate (80 mM), and L-lysine (80 mM)) were added to start the reactions. Measurements were carried out in 100 mM potassium phosphate buffers and metabolites. The instrumental parameters for the DMX-600 instrument were as follows: operating frequency, 600.13 MHz; spectral width, 6,009.61 Hz; memory size, 16 K; acquisition time, 1.363 s; number of transients, 48 to 144; pulse angle, 50° (3 μs); and relaxation delay with solvent presaturation, 1.6 s. Spectral resolution was enhanced by Gaussian multiplication with line broadening of –1 Hz and a Gaussian broadening factor of 0.19.

The time-evolution data for substrates and products were obtained by acquiring sequential spectra of the reactions. Progress curves were obtained by measuring the integrals of substrate resonances at each time point. Maximal rates were calculated from good fits (correlation coefficients of 0.99) of the data to straight lines for 30 to 80 min of incubation. Calibrations of the peaks arising from substrates were performed by extrapolating the resonance intensity data to zero time and assigning to this intensity the appropriate concentration value. The coding region of rocF is +1 to +969. Negative numbers refer to upstream residues. NA, not applicable.

### Table 1. Oligonucleotide primers, plasmids, and bacterial strains used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide primer, plasmid, or strain</th>
<th>Coordinates in rocF</th>
<th>Relevant genotype or description</th>
<th>DNA sequence (5′ to 3′)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROCF-F3 (for)</td>
<td>−149—−130</td>
<td>ap*, 1.154-bp rocF PCR product (nucleotides −149 to 1006) cloned into the PetI and ClaI sites</td>
<td>GTCTGCAATGTTGGGTTTCTATC</td>
<td>Stratagene This study</td>
</tr>
<tr>
<td>ROCF-R13 (for)</td>
<td>18—37</td>
<td>PetI site underlined</td>
<td>AACTGCAAGCAGAATGAGAGCG</td>
<td>N/A</td>
</tr>
<tr>
<td>ROCF-F4 (for)</td>
<td>204—223</td>
<td>ClaI site underlined</td>
<td>CGGATCCGATGGCCTGAAATAC</td>
<td>N/A</td>
</tr>
<tr>
<td>ROCF-R14 (rev)</td>
<td>339—360</td>
<td>BamHI site underlined</td>
<td>TCGGCTGTTGGGGCTC</td>
<td>N/A</td>
</tr>
<tr>
<td>ROCF-R6 (rev)</td>
<td>554—573</td>
<td>PetI site underlined</td>
<td>GTGCAATCAGAACAGAGAGAGAGATG</td>
<td>N/A</td>
</tr>
<tr>
<td>ROCF-R15 (for)</td>
<td>562—580</td>
<td>BamHI site underlined</td>
<td>CGGATCCGATGGCCTGAAATAC</td>
<td>N/A</td>
</tr>
<tr>
<td>ROCF-R17 (rev)</td>
<td>691—708</td>
<td>PetI site underlined</td>
<td>CTGCAATCAGAACAGGGAAGATG</td>
<td>N/A</td>
</tr>
<tr>
<td>ROCF-R16 (rev)</td>
<td>898—915</td>
<td>BamHI site underlined</td>
<td>CGGATCCGATGGCCTGAAATAC</td>
<td>N/A</td>
</tr>
<tr>
<td>ROCF-R5 (rev)</td>
<td>987—1006</td>
<td>PetI site underlined</td>
<td>GGATGCATTTTTTCAACTG</td>
<td>N/A</td>
</tr>
<tr>
<td>KanH16 (ev)</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KanK4 (rev)</td>
<td>NA</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>KanK5 (for)</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KanK8 (for)</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Plasmids (parent)

- pBluescript II SK (−)
- pBS-rocF::aphA3 (pBluescript II SK)
- pBS-rocF::aphA3 (pBluescript II SK)
- pHPl570 (pUC19)
- pILL570 Not (pILL570 Not)
- pILL570 (pILL570 Not)
- pILL600 (pILL600 Not)
- pILL235 (pILL570 Not)
- pILL236-2 (pILL570)

### Strains

- E. coli DH5α F− supE44 ΔlacU169 (Δ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1
- H. pylori
  - 26695 WT, genome sequenced
  - 26695 rocF::aphA3 mutant
  - N6 WT, naturally transformable laboratory strain
  - N6 236-2
  - N6 rocF::aphA3 mutant
  - SS1 WT (mouse-adapted)
  - SS1 236-2
  - SS1 rocF::aphA3 mutant

### Determination of cellular viability of H. pylori cells exposed to various pH buffers and metabolites

Suspensions of H. pylori 26695 and its isogenic rocF::aphA3 mutant were adjusted to an OD600 of ∼1.5 (10^8 to 10^9 viable cells/ml)
symbols

30 min. Data are presented as log 10 CFU killed by 30 min under the conditions start of the assay (under microaerobic conditions for 4 to 5 days. Viable CFU were plated prior to and plated for enumeration of viable CFU on blood agar. Plates were incubated resuspended in 0.9% NaCl, and the samples were serially diluted in 0.9% NaCl viability under these conditions. After incubation, incubation period. In control experiments at neutral pH, Bacteria were incubated at 37°C for 30 min in a microaerobic environment with a Campy Pouch (Becton Dickinson). The buffers did not change pH after this incubation, or without urea (5 to 20 mM), L-arginine (10 mM), or L-agmatine (10 mM).

Bacteria were incubated at 37°C for 30 min in a microaerobic environment with

H. pylori

cells were washed and resuspended in prewarmed 0.1 M citrate-phosphate buffer at a pH of 2.3 or 6.0 with

or without urea (5 to 20 mM), L-arginine (10 mM), or L-agmatine (10 mM).

H. pylori

mutants SS1

and SS1 236-2. All strains had undergone exactly 24

tions of animals. Mice (n = 9 to 11/group) were each inoculated orogastrically by using

polyethylene catheters with 100-

l) of urea-broth medium and peptone trypsin broth (Organotechnique, La Courneuve, France). The presence of urease activity in tissue fragments was determined by monitoring the urea-broth at room temperature for a color indicator shift to an alkaline pH as a result of the production of ammonium from urea hydrolysis. To perform quantitative bacterial cultures on stomach samples, tissue fragments were homogenized by using disposable plastic grinders and tubes (PolyLabo, Strasbourg, France). Quantitative culture was performed on homogenized samples, as previously described (7). The homogenates were serially diluted in sterile 0.85% NaCl and plated onto blood agar plates, supplemented with 200 μg of bacitracin and 10 μg of naladixic acid (Sigma Chemical Co.) per ml. After 3 to 4 days of incubation, colonies with

H. pylori

morphology were counted. The CFU data were statistically analyzed

by the Alternative Welch's t test, whereas colonization data were analyzed by using chi-squared analysis with the Fisher's exact test.

RESULTS


Previous results indicated that H. pylori has arginine activity and a urea cycle (21, 23). The H. pylori sequenced genomes analyses as previously described (7). Briefly, stomachs were washed in 0.85% (wt/vol) NaCl and dissected longitudinally into two sections. Fragments of each stomach were placed into aliquots (400 μl) of urea-broth medium and peptone trypsin broth (Organotechnique, La Courneuve, France). The homogenates were serially diluted in sterile 0.85% NaCl and plated onto blood agar plates, supplemented with 200 μg of bacitracin and 10 μg of naladixic acid (Sigma Chemical Co.) per ml. After 3 to 4 days of incubation, colonies with H. pylori morphology were counted. The CFU data were statistically analyzed by the Alternative Welch's t test, whereas colonization data were analyzed by using chi-squared analysis with the Fisher's exact test.

RESULTS


Previous results indicated that H. pylori has arginine activity and a urea cycle (21, 23). The H. pylori sequenced genomes

TABLE 2. Strategy used for verification of rocF mutants by PCR and sequence analyses

<table>
<thead>
<tr>
<th>Strain</th>
<th>PCR primer pairs(a)</th>
<th>PCR-sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Up, bp(^b)</td>
<td>Down, bp</td>
</tr>
<tr>
<td>N6 WT</td>
<td>R13 + K4, none</td>
<td>R5 + K8, none; R17 + K8, none</td>
</tr>
<tr>
<td>N6 rocF::aphA3</td>
<td>R13 + K4, 680</td>
<td>R5 + K8, 860</td>
</tr>
<tr>
<td>N6 236-2</td>
<td>R13 + K4, 890</td>
<td>R17 + K8, 250</td>
</tr>
<tr>
<td>SS1 WT</td>
<td>R13 + K4, none</td>
<td>R17 + K5, none; R17 + K8, none</td>
</tr>
<tr>
<td>SS1 rocF::aphA3</td>
<td>R13 + K4, 680</td>
<td>R17 + K5, 1160</td>
</tr>
<tr>
<td>SS1 236-2</td>
<td>R13 + K4, 890</td>
<td>R17 + K8, 250</td>
</tr>
</tbody>
</table>

\(^{a}\) Primer pairs used for PCR are listed. Primers are schematically depicted in Fig. 1. The first primer of the pair is specific for rocF, whereas the second primer is specific for the kanamycin resistance gene. The primer sequences are given in Table 1. The results for the PCR reactions from columns 2 and 3 of the table are shown in Fig. 2B.

\(^{b}\) Up and down, rocF regions immediately up- or downstream, respectively, of insertion sites for kanamycin cassettes. The sizes of the PCR products (shown in Fig. 2B) are given in base pairs.

\(^{c}\) NA, not applicable.

\(^{d}\) ND, not determined.

\(^{e}\) Underlining indicates primers used for direct sequencing of PCR products.
revealed genes hp1399 and hp1427 in strains 26695 and J99, respectively, as predicted orthologs of arginase (1, 34). To investigate the functions of rocF in vitro and in vivo, the rocF gene was cloned by PCR, sequenced, and found to be identical to the previously published sequence of strain 26695 (34). The 969-bp coding region of rocF does not appear to be cotranscribed with any other gene based on the finding that the upstream gene (hp1398) is in the opposite orientation to that of rocF, and the 5’ end of the downstream gene (hp1400) is more than 500 bp away from the 3’ end of rocF (1, 34; see also below). Additionally, rocF has a predicted near consensus rho-independent transcriptional terminator (CUUUUCAAAACC N11GGUUGAAAAAG), followed by an AU-rich region. The rocF gene has a predicted Shine-Dalgarno sequence of TAA GGAGGTG that closely matches the consensus (TAAAGGA GT) and two strong predicted s30-like promoters.

The predicted pI for H. pylori RocF is 6.4, and Leu, Lys, and Gin residues comprise 30% of the protein. The predicted RocF protein has the three conserved histidine residues (H91, H118, and H133), including the one within the putative divalent cation-binding motif D-A-H-X-D (amino acids 116 to 122) found in all agmatinases and arginases.

Construction and confirmation of rocF allelic exchange mutants of H. pylori. Allelic exchange rocF mutants of H. pylori were constructed (Fig. 1; Table 1) by using two different strategies and three different H. pylori strains. To confirm the construction of rocF mutants of H. pylori, chromosomal DNA from WT and mutants was analyzed by Southern blot, PCR, and PCR sequencing.

Southern blot analyses of AvaI-digested chromosomal DNA from H. pylori 26695 WT and rocF::aphA3 mutant strains, when probed with a 240-bp fragment of the rocF gene, resulted in probe-positive fragments with sizes of 2.2 and 3.4 kb, respectively (Fig. 2A). The aphA3 probe also hybridized to a 3.4-kb AvaI fragment from chromosomal DNA from the mutant but did not hybridize with WT chromosomal DNA (Fig. 2A), thus confirming the insertion of the kanamycin cassette in the rocF gene of H. pylori 26695.

The rocF mutants of H. pylori strains N6 and SS1 were confirmed by PCR and PCR-sequencing analyses by using the strategies outlined in Table 2. Primers were designed to amplify DNA fragments corresponding to the sequences both upstream and downstream of the insertion sites of the kanamycin cassettes (Fig. 1). The expected PCR product sizes were obtained in all cases (Fig. 2B; Table 2 shows the fragment sizes). As expected, no PCR products were obtained with any primer pair when using chromosomal DNA from any of the WT H. pylori strains (Fig. 2B, lanes 1, 4, 7, 9, 11, and 13). Additionally, PCR analysis of the hp1400 gene downstream of rocF in the rocF mutants with an hp1400 (fecA)-specific primer plus a rocF primer yielded products with the appropriate size in all of the strains tested (data not shown). Direct sequence analysis of selected PCR products (Table 2) provided unequivocal evidence that the junctions of rocF and aphA3 were correct in these H. pylori mutant strains.

H. pylori rocF mutants retain WT levels of urease activity. The rocF mutants of H. pylori had similar rates of growth in broth to those of the WT strains (data not shown). To ascertain whether arginase affects urease activity in H. pylori, we measured this enzyme activity in the WT and rocF::aphA3 mutant of H. pylori 26695. No significant difference was observed in the urease activities, with averages of 4,400 ± 200 and 4,500 ± 500 nmol of ammonium/min/mg of protein for the WT and rocF::aphA3 mutant, respectively (n = 3; P = 0.69). Similarly, no

FIG. 2. Confirmation of rocF allelic exchange mutants of H. pylori. (A) Southern blot analysis of H. pylori WT and the rocF::aphA3 isogenic mutant. Chromosomal DNA (~6 μg) from H. pylori 26695 and 26695 rocF::aphA3 was digested with AvaI and processed as described in Materials and Methods. The blot was probed with either the kanamycin cassette (1.2 kb) or a portion of the rocF gene (240-bp HindIII fragment). Note that when probed with rocF, the mutant has an increase in molecular weight by ~1.2 kb, as expected. (B) PCR analysis of rocF mutants of H. pylori. The primer pair strategies used are shown in Table 2. Lanes (number, H. pylori strain, primer pair): 1, N6 WT, R13 and K4; 2, N6 rocF::aphA3, R13 and K4; 3, N6 236-2, R13 and K4; 4, SS1 WT, R13 and K4; 5, SS1 rocF::aphA3, R13 and K4; 6, SS1 236-2, R13 and K4; 7, N6 WT, R5 and K8; 8, N6 rocF::aphA3, R5 and K8; 9, N6 WT, R17 and K8; 10, N6 236-2, R17 and K8; 11, SS1 WT, R17 and K5; 12, SS1 rocF::aphA3, R17 and K5; 13, SS1 WT, R17 and K8; 14, SS1 236-2, R17 and K8; and M, 1-kb marker (Gibco BRL).
Table 3. Rates of amino acid catabolism determined for *H. pylori* cells of WT and rocF mutant strains (n = 4)\(^a\)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean rate of enzyme activity (nmol/min/mg of protein) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arginase</td>
</tr>
<tr>
<td>26695</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>26695 rocF::aphA3</td>
<td>UD</td>
</tr>
<tr>
<td>N6</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>N6 rocF::aphA3</td>
<td>UD</td>
</tr>
<tr>
<td>N6 236-2</td>
<td>UD</td>
</tr>
<tr>
<td>SS1</td>
<td>54 ± 5</td>
</tr>
<tr>
<td>SS1 rocF::aphA3</td>
<td>UD</td>
</tr>
<tr>
<td>SS1 236-2</td>
<td>UD</td>
</tr>
</tbody>
</table>

\(^a\) Bacteria were suspended in a phosphate (20 mM; pH 7)–NaCl (115 mM)–KCl (15 mM) buffer plus one of the following amino acids: L-arginine (100 mM), L-asparagine (80 mM), L-aspartate (80 mM), L-glutamine (80 mM), or l-serine (80 mM) and were measured for enzyme activity by NMR (see Materials and Methods).

\(^b\) UD, undetectable. \(^P < 0.0001\) compared with its isogenic WT strain.

\(^c\) \(^P < 0.05\) compared with its isogenic WT strain.

\(^d\) \(^P < 0.05\) compared with its isogenic WT strain.

\(^e\) \(^P < 0.01\) compared with its isogenic WT strain.

H. pylori rocF mutants lack arginase activity. Catabolism of l-arginine by *H. pylori* was investigated in cell suspensions of the WT strains 26695, N6, and SS1 and the corresponding rocF mutants by employing the \(^1\)H-NMR spectroscopy method described previously (23). All three WT strains of *H. pylori* expressed arginase activity (Table 3). All five rocF mutants constructed were completely devoid of arginase activity (Table 3).

Asparaginase, aspartase, glutaminase, and serine dehydratase activities in WT and rocF mutants of *H. pylori*. To determine whether the inactivation of rocF could result in alteration of other enzyme activities involved in nitrogen metabolism, the deamination rates of l-asparagine, l-aspartate, l-glutamine, and l-serine were determined in *H. pylori* cell suspensions of the WT and rocF mutants by employing \(^1\)H-NMR spectroscopy (20). Fast rates of asparaginase, aspartase, glutaminase, and serine dehydratase enzyme activities were observed for the WT strains (Table 3). No significant differences were observed between WT *H. pylori* and their corresponding rocF mutants in asparaginase, aspartase, and glutaminase activities (Table 3). Most notably, however, there was a significant decrease in serine dehydratase activities in all rocF mutants relative to the WT strains (Table 3). Additionally, the activities of the rocF::aphA3 mutants were lower than those of the 236-2 mutants (Table 3). To determine whether these differences arose from modulation of enzyme activity by ammonium ions, serine dehydratase activities were measured in lysates containing NH\(_4\)Cl at concentrations of up to 15 mM. The presence of NH\(_4\)Cl did not alter the rates of enzyme activity (data not shown).

Effect of RocF on survival of *H. pylori* from acid exposure. Urease mutants of *H. pylori* are much more sensitive to acid in vitro than are WT strains due to loss of hydrolysis of urea to acid-neutralizing ammonium (3a, 15, 31, 33). Since arginine is a precursor source of urea for *H. pylori* through arginase activity (21), we investigated the role of *H. pylori* arginase activity in sensitivity of *H. pylori* to acid. *H. pylori* 26695 and its isogenic rocF::aphA3 mutant were incubated for 30 min in citrate-phosphate buffer at pH 2.3 or 6.0 under microaerobic conditions in the presence or absence of urea, arginine, or agmatine. At pH 2.3, although the WT was sensitive to acid (viability was reduced by about 5 orders of magnitude), the rocF mutant was ~1,000-fold more sensitive to acid (viability reduced by about 8 orders of magnitude) (Fig. 3). Urea at a concentration of 5 mM added to citrate-phosphate buffer at pH 2.3 increased the viability of both strains, with a reduction in viable bacteria (relative to *T*\(_0\) counts) of only 2.4 and 2.9 orders of magnitude, respectively (Fig. 3). Urea at a concentration of 20 mM added to citrate-phosphate buffer at pH 2.3 further increased the viability of both strains (Fig. 4). As a control, addition of urea to citrate-phosphate buffer at pH 6.0 had no effect on the cell viability of either strain (Fig. 3). These results indicated that the rocF::aphA3 mutant was remarkably more susceptible to acid than the WT and that both strains survived equally well when exogenous urea was added.

![Image](http://jb.asm.org/Downloaded_from http://jb.asm.org/ on September 9, 2017 by guest)
The survival of the WT and rocF::aphA3 mutant in the presence of arginine or agmatine (10 mM) was subsequently investigated. Arginine dramatically increased the viability of the WT strain at pH 2.3, with a reduction of viable bacteria (relative to T₀ counts) of approximately 6 and 4 orders of magnitude in the absence versus the presence of arginine, respectively. In contrast, the addition of arginine did not increase the viability of the rocF::aphA3 mutant in acidic conditions, with similar reductions in viable bacteria, irrespective of the absence or presence of arginine (Fig. 4). Thus, there was a ~10,000-fold decrease in the viability of the rocF::aphA3 mutant compared with the WT under acid conditions in the presence of arginine. This decrease in the viability of the rocF::aphA3 mutant compared with the WT under acid conditions plus arginine was maintained in a dose-dependent fashion (data not shown), and maximal protection from acid of both strains was observed at 50 mM arginine, a concentration known to be near saturating for arginase activity (21). As a control, the addition of arginine to citrate-phosphate buffer (pH 6.0) had no effect on the viability of either the WT or rocF::aphA3 mutant (data not shown). Agmatine did not protect either the WT or mutant from acid exposure (Fig. 4). At an intermediate pH (4.2), the viabilities of the WT and the rocF::aphA3 mutant were similar, as expected: in two experiments, each conducted in duplicate, reductions of viable bacteria by 2.8 and 3.5 orders of magnitude were observed (data not shown). This viability was intermediate to that of strains tested at pH 2.3 and 6.0.

_H. pylori rocF is not required for colonization of mice._ Since urease and arginase mutants both exhibit increased acid sensitivity in vitro (this study and references 3a, 15, 31, and 33) and urease is required for _H. pylori_ colonization in vivo (4, 5), we investigated whether _H. pylori_ arginase is required for colonization. Mice were challenged with equivalent inocula of WT _H. pylori_ SS1 and the two rocF isogenic mutants. In mutant SS1 rocF::aphA3, rocF was disrupted by insertion of a kanamycin resistance cassette. In mutant SS1 236-2, rocF was disrupted by deletion of an internal rocF fragment and replaced with a kanamycin resistance cassette (Fig. 1 and 2). At 1 month postinoculation, 89% (8 of 9) of mice administered the _H. pylori_ SS1 WT isolate were colonized with the organism, whereas 40% (4 of 10) and 82% (9 of 11) of animals that had been challenged with either _H. pylori_ rocF::aphA3 and 236-2 mutants, respectively, were infected (P = 0.06 and P = 1.0, respectively, compared with the WT) (Fig. 5). Quantitative culturing on nonselective plates revealed similar numbers of viable _H. pylori_ CFU per gram of tissue for the WT and mutant 236-2, with a mean log₁₀ CFU/g of tissue ± standard deviation of 6.1 ± 1.4 and 5.5 ± 1.3, respectively (P = 0.36). In contrast, bacterial loads in mice challenged with the rocF::aphA3 mutant were significantly lower, with a 4.4 ± 1.8 log₁₀ CFU/g of tissue (P = 0.03) (Fig. 5), a finding in agreement with its reduced colonization. Quantitative culturing on kanamycin-containing plates of gastric tissues from mice that had been administered either of the _H. pylori_ SS1 rocF mutants revealed results similar to those obtained on nonselective plates, thus demonstrating the stability of the kanamycin cassettes in vivo (data not shown). Urease activities of the rocF mutants of _H. pylori_ recovered from mice were similar to those of the WT strain and to those of the rocF mutant used to inoculate the mice (data not shown).

In a second independent experiment (data not shown in Fig. 5), the numbers of mice colonized with the WT, 236-2 rocF mutant, and rocF::aphA3 mutant were 64% (7 of 11), 100% (10 of 10), and 56% (5 of 9), respectively, a result in good agreement with the results of the first mouse experiment.

**DISCUSSION**

The _H. pylori_ rocF gene (34) was cloned, and five isogenic rocF mutants with disruptions in this gene were constructed in three strains and were shown to completely lack arginase activity and to have reduced serine dehydratase activity (Table 3). Additionally, the rocF::aphA3 mutant of strain 26695 was dramatically more sensitive to acid and could not be rescued from acid exposure by addition of exogenous arginine, in comparison with the WT. Based on these results and the homology of RocF with the arginase family (1, 23, 34), it is concluded that rocF encodes the _H. pylori_ arginase of the bacterium. _H. pylori_ rocF mutants colonized mice, albeit to different degrees depending on the rocF disruption strategy.

_H. pylori_ rocF mutants had urease activities similar to those of WT _H. pylori_ in vitro. Exogenous urea protected the WT and the rocF::aphA3 mutant of strain 26695 equally well from acid, indicating that _H. pylori_ arginase has no effect on urease activity under the conditions tested. Conversely, a urease mutant of _H. pylori_ N6 was previously shown to have the same arginase activity as the WT strain (23). Notwithstanding the apparent close metabolic relationship between arginase and urease, in _H. pylori_ these enzymes did not appear to influence the activity of each in vitro.

It was demonstrated that arginase activity plays a critically important role in the survival of _H. pylori_ from acid exposure in vitro because the WT, but not the rocF::aphA3 mutant, can be rescued from acid exposure by exogenous addition of arginine (Fig. 3 and 4). Agmatine had no effect on the survival of _H. pylori_ from acid exposure, indicating that the activity being observed is arginase, not agmatinase. The rocF::aphA3 mutant of _H. pylori_ 26695 was ~1,000-fold more sensitive to acid exposure in vitro than the WT strain. The increased sensitivity of the rocF^−^ mutant to acid may arise from its inability to synthesize urea from arginine, which would result in a deficiency of acid-neutralizing ammonia available from urease activity. However, other urease-independent mechanisms of ammonia generation also could be involved in acid protection of _H. pylori_, since _H. pylori_ exhibits several active amino acid deaminases (Table 3 and reference 20) and one or more aliphatic amidases (32). Preliminary experiments, however, suggested that the amino acid deaminases play little or no role in protecting _H. pylori_ from acid exposure (19a). The reason for the increased viability of _H. pylori_ treated in acid conditions plus

![FIG. 5. In vivo colonization of mice by WT and rocF mutants of _H. pylori_ SS1. Bacterial loads were enumerated by quantitative culture on samples obtained 1 month postinoculation. The limit of detection is indicated by the dotted line. Symbols along the dotted line indicate that the strain failed to colonize the mouse. Urea-positive (solid symbols) and negative (open symbols) gastric biopsy samples are also shown. Each point corresponds to a determination for a single mouse. Solid lines indicate the mean log₁₀ of viable CFU per gram of tissue. In a second experiment (quantitative data not shown in the figure), the numbers of mice colonized with the WT, 236-2 rocF mutant, and rocF::aphA3 mutant were 64% (7 of 11), 100% (10 of 10), and 56% (5 of 9), respectively.](http://jb.asm.org/Downloaded/from/http://jb.asm.org/)
urea compared with those treated with arginine could be due to differences in affinities of specific transporters for urea versus arginine or to the WT strain’s ability to synthesize its own urea by arginase activity but an inability to synthesize its own arginine.

\( H. \) pylori rocF mutants showed pleiotropic effects: in addition to the loss of arginase activity, serine dehydratase activities were markedly reduced in all mutants compared with the corresponding WT \( H. \) pylori, with stronger effects observed for the rocF::aphA3 mutants than for the 236-2 mutants (Table 3). Serine dehydratase deaminates serine to pyruvate plus ammonium; pyruvate has been shown to play an important role in the energy metabolism of \( H. \) pylori (22, 28). \( H. \) pylori rocF is at a locus distinct from the serine dehydratase locus (genes adaA, hp0132, and jhp0120 in \( H. \) pylori 26695 and 199, respectively) (1, 34). Although reduced serine dehydratase activities could be due to impaired systems of ammonium excretion in the rocF mutants, the mechanism of this effect and the differences in serine dehydratase activities in the rocF::aphA3 and 236-2 mutants remain to be elucidated, possibly with the help of \( H. \) pylori isogenic serine dehydratase mutants. However, the differences between the serine dehydratase activities of the SS1, SS1 236-2, and SS1 rocF::aphA3 strains suggested a relationship between the metabolic and mouse colonization data; the lower serine dehydratase enzyme rates of the SS1 rocF::aphA3 mutant may correlate with its less-effective colonization than the SS1 236-2 mutant. The in vivo data indicated that \( H. \) pylori arginase activity and the rocF gene are not essential for colonization of mice, since the 236-2 rocF mutant of \( H. \) pylori SS1 colonized mice as well as the WT strain. Interestingly, however, the rocF::aphA3 H. pylori mutant had a moderately reduced colonization efficiency, as demonstrated by the finding that only 40% of mice that were challenged with this mutant were still infected at 1 month postinoculation (Fig. 5). Arginase may only be necessary in vivo in situations such as limiting urea concentrations in the gastric mucosa, lowered host arginase levels, or lowered stomach pH. \( H. \) pylori rocF mutants may be able to colonize mice because the murine stomach has a slightly higher pH than that of humans, and in this changed environment arginase may not be essential to protect \( H. \) pylori from acid. Another reason for colonization by rocF mutants could be that host arginase activity compensated for arginase deficiency in the rocF mutants. Host arginase could provide the required urea concentrations to rocF mutants of \( H. \) pylori in vivo to generate acid-neutralizing ammonium from urease. Unfortunately, this hypothesis cannot be readily tested because host arginase activity cannot be inhibited without deleterious effects (2a).

It is unlikely that insertion of the kanamycin-resistance cassette into rocF causes polar effects on other genes adjacent to rocF, since there is a strong predicted transcriptional terminator downstream of the coding region of rocF and the translation start site for the gene downstream of rocF, hp1400, is more than 500 bp away from the 3’ end of rocF (1, 34). Also, the kanamycin cassettes used for construction of the rocF mutants lack a transcriptional terminator, thereby minimizing premature termination of rocF transcription. Additionally, the rocF mutants were vigorously confirmed by multiple techniques to rule out potential artifacts (Table 2 and Fig. 2). Thus, polar effects are probably not the reason for the colonization and serine dehydratase activity differences observed between the two rocF mutants. These differences, as well as the pleiotropic phenotypes of the rocF mutants, suggest that caution should be exercised in interpreting results of gene knockout experiments when only one allelic exchange mutant is used. Finally, it is highly unlikely that the decrease in serine dehydratase activity is due to spontaneous mutation of this locus because we have constructed five different arginase mutants in three different strains of \( H. \) pylori. The chances of a spontaneous mutation in this specific locus happening five independent times would be lower than \( 1 \times 10^{18} \) (1,600 genes raised to the fifth power, assuming one phenotypic mutation per genome). Also the WT and mutant strains were passaged an equal number of times in vitro. All of the corresponding isogenic WT strains have WT rates of serine dehydratase activity even after several in vitro passages.

In summary, the \( H. \) pylori rocF gene encodes the urea cycle enzyme arginase and is required for arginase activity. A rocF mutant of \( H. \) pylori has increased susceptibility to acid treatment in vitro, and thus arginase activity dramatically helps \( H. \) pylori survive acid exposure in an arginine-dependent manner. rocF mutants also have decreased serine dehydratase activity, retain WT levels of urease activity, and are able to colonize mice but to different degrees. The data suggest that \( H. \) pylori and/or the host have a mechanism for in vivo compensation of the loss of the bacterial arginase.

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