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In Vitro and In Vivo Complementation of the *Helicobacter pylori* Arginase Mutant Using an Intergenic Chromosomal Site

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Abstract

Background—Gene complementation strategies are important in validating the roles of genes in specific phenotypes. Complementation systems in *Helicobacter pylori* include shuttle vectors, which transform *H. pylori* at relatively low frequencies, and chromosomally based approaches. Chromosomal complementation strategies are susceptible to polar effects and disruption of other *H. pylori* genes, leading to unwanted pleiotropic effects.

Materials and methods—A new complementation strategy was developed for *H. pylori* by utilizing a suicide plasmid vector that contains fragments of an *H. pylori* intergenic region (*hp0203–hp0204*), a chloramphenicol acetyltransferase cassette (*cat*), and a multiple-cloning site. Genes of interest could be cloned into the intergenic plasmid and the genes integrated into *H. pylori* by homologous recombination into the intergenic chromosomal region without disrupting any annotated *H. pylori* gene. The complementation system was validated using the gene encoding arginase (*rocF*).

Results—A *rocF* mutant unable to hydrolyze or consume L-arginine regained these functions by complementation with the wild-type *rocF* gene. Complemented strains also had restored arginase protein as determined by Western blot analysis. The complementation system could be successfully applied to multiple *H. pylori* strains. The intergenic region varied in length and sequence across 17 *H. pylori* strains, but the flanking-3' ends of the *hp0203* and *hp0204* coding regions were highly conserved. Inserting a *cat* cassette and wild-type *rocF* into the intergenic region did not alter the ability of strain SS1 to colonize mice.

Conclusions—This complementation strategy should greatly facilitate genetic experiments in *H. pylori*.

Keywords

Helicobacter pylori; arginase; complementation

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Since its discovery in 1982 by Marshall and Warren [1], *Helicobacter pylori* has been shown to be the causative agent of chronic gastritis, gastric and duodenal ulcers, and mucosa-associated lymphoid tissue (MALT) lymphoma [2,3]. Many infected individuals fail to develop clinical symptoms of disease, although all exhibit histologic evidence of pathology [4,5]. However, the exact mechanisms employed by *H. pylori* to cause such varied host pathology is unknown.

Research on *H. pylori* pathogenesis has been partially hampered by insufficient and suboptimal complementation systems. Complementation is the process of introducing a wild-type copy of a gene into a mutant strain disrupted for the same gene, leading to restoration of phenotypes that were lost in the mutant. Current methods used for gene complementation in *H. pylori* include shuttle plasmids and chromosomally based systems. Shuttle plasmids for *H. pylori* are hybrids comprised of *H. pylori* cryptic plasmids and plasmids isolated from *Escherichia coli*, and they contain antibiotic resistance markers for selection [6–10]. Unfortunately, these shuttle plasmids transform some *H. pylori* strains at low frequencies and completely fail to transform other *H. pylori* strains [6,8]. Chromosomally based complementation systems in *H. pylori* have been used to complement genes such as *luxS*, *recG*, *rdxA*, *comH*, and *ureB*, but these systems interrupt important chromosomal segments or can be susceptible to polar effects that may influence expression of other *H. pylori* genes [11–15]. Therefore, improved genetic complementation tools are needed for confirming the function of *H. pylori* genes.

This study was undertaken to develop a different complementation system and was validated by complementing the gene encoding *H. pylori* arginase, *rocF*. Arginase is an enzyme that hydrolyzes L-arginine to L-ornithine and urea. The urea is then hydrolyzed by urease to produce CO₂ and ammonia, the latter serving to neutralize harmful stomach acid for *H. pylori* [16, 17]. Arginase-mediated hydrolysis of arginine consumes the same substrate required for nitric oxide (NO) synthesis [18], an important innate host defense mechanism. The consumption of host L-arginine by *H. pylori* arginase can lead to dysregulation of the host immune response through inhibition of T-cell proliferation and CD3 ζ chain expression on the T-cell surface [19].

Herein, we describe a gene complementation system that utilizes a suicide plasmid vector containing a specific intergenic region (between genes *hp0203* and *hp0204*) from the *H. pylori* chromosome. These regions were separated into two fragments, IR203 and IR204, flanking a multicloning site. Once introduced into the bacterium by electroporation, these intergenic regions can undergo double cross-over homologous recombination into the bacterial chromosome, thereby inserting any genes cloned within the multicloning site and eliminating extraneous plasmid sequences. IR203 and IR204 regions were initially selected because the region represents one of the largest intergenic regions in the *H. pylori* genome. Moreover, this region is reasonably well conserved between the sequenced *H. pylori* strains (26695 and J99) [20,21]. Additionally, the *hp0203* and *hp0204* hypothetical genes are adjacent in 26695 and J99 strains. The *hp0203* and *hp0204* genes are transcribed towards each other, greatly minimizing possible polar effects, and avoiding insertion of DNA fragments into putative promoter regions. Finally, no annotated *H. pylori* genes will be disrupted by this chromosomally based strategy.

By using this plasmid vector system, the arginase mutant was complemented for the first time in *H. pylori*, and phenotypic effects lost in the *rocF* mutant *H. pylori* could be restored via complementation as determined by arginase activity, Western blot, and L-arginine consumption. It was also shown that the complementation system can be used for in vivo experiments without supplying antibiotics to animals. This technology provides a general method for complementing *H. pylori* mutants.

Materials and Methods

Bacterial Strains and Growth Conditions

All *H. pylori* strains were grown on Campylobacter blood agar (Becton, Dickinson, & Co., Sparks, MD, USA) containing 10% sheep blood (Quad Five, Ryegate, MT, USA), at 37 °C, 10% CO₂, 5% O₂, and 85% N₂ atmospheric conditions for 48 hours, unless otherwise stated. For animal experiments *H. pylori* strains were grown overnight in Mueller–Hinton broth plus 2% fetal bovine serum (FBS) without aeration under the same atmospheric conditions as above. *E. coli* strains were grown on Luria agar or broth at 37 °C overnight. Concentrations of antibiotics used for *H. pylori* were kanamycin (15 µg/ml) and chloramphenicol (10 µg/ml). Antibiotics for *E. coli* strains were used at the following concentrations: ampicillin (100 µg/ml); kanamycin (25 µg/ml); and chloramphenicol (20 µg/ml). Complete listings of bacterial strains used in this study are in Table 1.

Molecular Biology

Plasmid DNA was extracted by either the alkaline lysis method [22] or the column chromatography method (QIAGEN, Valencia, CA, USA). *H. pylori* chromosomal DNA was isolated by suspending *H. pylori* in 480 µl lysis buffer (10 mmol/l Tris, 75 mmol/l NaCl, 25 mmol/l ethylenediamine tetra-acetic acid, 1% SDS, pH 7.8) plus proteinase K (300 µg/ml) and incubated at 55 °C overnight, followed by phenol–chloroform and chloroform extractions and then ethanol precipitation. DNA was resuspended in 50 µl 10 mmol/l Tris-HCl (pH 8.5) plus RNase (10 µg/ml). Restriction enzymes were from Promega (Madison, WI, USA). Ligations were performed using a Rapid DNA Ligation kit (Roche, Indianapolis, IN, USA). DM3-DM6 primers were obtained from the Biopolymer Laboratory at the University of South Alabama (Mobile, AL, USA). All other primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Primer sequences are listed in Table 2.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) conditions were set at 30 cycles, with one cycle consisting of 94 °C for 30 seconds, 55–60 °C for 30 seconds, and 72 °C for 30 seconds with *Taq* DNA polymerase (Promega, Madison, WI, USA). Complemented strains of *H. pylori* were confirmed with the following oligonucleotides: *cat*-F2, *kan*-F2, *rocF*-F25, *rocF*-R25, and *hp0203*-F1 (Table 2). Amplification and sequence analysis of the intergenic plus adjacent regions was accomplished in different *H. pylori* strains using primers DM71 and DM189 (Table 2).

Southern Hybridization

Chromosomal DNA from *H. pylori* strains was digested with *Bam*HI and *Eco*RI overnight. DNA was transferred to Hybond-N⁺ nylon membrane (Amersham Biosciences, Piscataway, NJ, USA). An ECL Direct Nucleic Acid Labeling and Detection System (Amersham) was used for labeling a 3.0 kb gel-purified pBluescript II SK(–) (pBS) (Stratagene, LaJolla, CA) fragment digested with *Eco*RV and *Pst*I. Blots were washed stringently and treated according to the manufacturer's specifications.

Construction of Plasmid Vectors (Table 2)

All plasmids were confirmed by restriction analysis and DNA sequencing (Iowa State University Sequencing Facility, Ames, IA, USA).

pIR204

The IR204 region (Fig. 1A) was PCR-amplified from *H. pylori* 26695 using primers DM5 and DM6 and digested with *Xho*I and *Kpn*I, and the 326 bp fragment was cloned into pBS digested with *Xho*I and *Kpn*I.

pIR20304

The pIR204 plasmid and PCR-amplified IR203 DNA (primers DM3 and DM4; 332 bp) were digested with *Sac*I and *Xba*I restriction endonucleases, gel-purified (QIAGEN, Valencia, CA, USA), and ligated to form pIR20304 (Fig. 1B).

pIR203C04 (Intergenic Plasmid)

pIR20304 was digested with *Hinc*II, and pBS-*cat* was digested with *Eco*RV and *Sma*I. After agarose gel electrophoresis, pIR20304 (3.66 kb) and a nonpolar *cat* (chloramphenicol acetyltransferase) cassette (0.84 kb) from pBS-*cat* (Table 1) were purified and the fragments ligated to form pIR203C04 (Fig. 1B). Restriction sites remaining for cloning additional fragments are: *Bam*HI, *Sma*I, *Eco*RV, and *Pst*I.

pIR203K04 (Intergenic Plasmid)

This plasmid is similar to pIR203C04, except a nonpolar kanamycin resistance cassette was cloned. pIR20304 was digested with *Hinc*II and pBS-kan (29) was digested with *Hinc*II and *Sma*I. The pIR20304 vector (3.66 kb) and kan cassette (1.25 kb) insert were purified with QIAquick and ligated to form pIR203K04. Restriction sites remaining for cloning are *Bam*HI, *Cla*I, *Hind*III, and *Sma*I.

pMLB001, pMLB002, pMLB003, and pMLB004

Four plasmids, pJH43504, pJHSS1, pJH63, and pBS-*rocF* (Table 1), contain the *rocF* gene cloned from *H. pylori* 43504, SS1, J63, and 26695, respectively. These plasmids were digested with *Bam*HI and *Hinc*II and the 1.1 kb *rocF* fragment was gel-purified and ligated into the *Bam*HI and *Sma*I sites of pIR203C04 (4.5 kb) to yield pMLB001, pMLB002, pMLB003, and pMLB004, respectively (Fig. 1B).

Transformation of *H. pylori* and *E. coli*

H. pylori was transformed with 3 µg plasmid or chromosomal DNA by electroporation (2.5 kV, 50 µF, 200 ohms, time constant = 10 ms). Electroporation typically reduces viable *H. pylori* counts by about two orders of magnitude. Following electroporation, bacteria were spotted onto Campylobacter blood agar (CBA) lacking antibiotics and grown for 2 days and then removed and placed on selective media. Transformation frequency was determined by dividing the number of transformed colonies per milliliter by total CFU/ml of bacteria postelectroporation (T_0). *E. coli* cells were transformed by the calcium chloride method [22].

Construction of *H. pylori* *rocF*/*rocF*⁺ Complemented Strains

The *rocF* mutant of *H. pylori* strain 26695 was transformed with pMLB001, pMLB002, pMLB003, or pMLB004 by electroporation, and transformants were selected on CBA agar containing chloramphenicol and kanamycin. Chromosomal DNA from transformed colonies was isolated and confirmation of each strain was performed by PCR analysis (see Results section; Fig. 2A–D). Southern blot was also used to eliminate the possibility that a single cross-over event occurred. Other genetically engineered *H. pylori* strains (Tables 1 and 3) were constructed and confirmed in the same fashion, except where noted. Confirmed strains were frozen at –80 °C in 1 ml Ham's F12 (Hyclone, Logan, UT, USA) broth plus 20% glycerol. As a control, both wild-type 26695 and the *rocF* mutant 26695 were transformed with the

pIR203C04 plasmid lacking *rocF*, selected on CBA plus chloramphenicol, and confirmed by PCR, as above.

Arginase Activity

Arginase activity of *H. pylori* and *E. coli* was determined as described previously [23]. Data are presented as mean specific activity $\pm$ standard deviation. All experiments were conducted at least twice with duplicate measurements, with a representative experiment shown.

Western Blot

Bacterial extracts containing arginase were prepared as described previously [23]. Protein (20 μ g) was loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and electrophoresed (125 V, 120 minutes). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA), incubated with anti-RocF (1: 3000) [23], and anti-rabbit IgG conjugated with alkaline phosphatase (1: 10,000, Sigma, St. Louis, MO, USA). Nitro-blue tetrazolium (100 μ g/ml) and 3-indoxyl phosphate (10 μ g/ml) were used as detection reagents.

L-Arginine Consumption by *H. pylori*

Experiments were conducted in RPMI 1640 medium plus 10% FBS. Bacteria were inoculated at 5×10^5 viable CFU and allowed to grow for 48 or 72 hours. The supernatant was retained for quantification of L-arginine concentration by high-performance liquid chromatography electrochemical detection (HPLC-ECD) as previously described [24], using an ESA-CoulArray Model 540 (ESA Inc, Chelmsford, MA, USA) and an 80×3.2 mm column with 120 Å pore size. Supernatants were briefly deproteinized by methanol. After centrifugation at $6000 \times g$ for 10 minutes at 4 °C, the supernatant was derivatized with 0.2 mol/l o-phthalaldehyde containing β -mercaptoethanol. Each sample (50 μ l) was injected into the column. The retention time for the L-arginine standard (prepared in methanol) was 10.2 minutes.

Colonization of Mice

Animal experiments were approved by the Institutional Animal Care and Use Committee at the University of South Alabama. Mice (129 Ev/Sv strain, in-bred internally) were orally inoculated with 50 μ l ($\sim 1 \times 10^9$ CFU/ml in 1 $\times$ phosphate-buffered saline [PBS]) of either wild-type SS1 (n = 8 animals), the *rocF* mutant SS1 (n = 7 animals), the wild-type carrying the *cat* cassette within the *hp0203–204* intergenic region (SS1 IR203C04; Table 1) (n = 4 animals) or the SS1 *rocF/rocF*⁺ (Table 1) carrying the *cat* cassette plus the wild-type *rocF* gene inserted into the *hp0203–hp0204* intergenic region (n = 5 animals). Mice were given antibiotic-free water and food *ad libitum*. At 1 week post-infection, animals were humanely euthanized. Stomachs were removed, dissected longitudinally along the greater curvature, and the chyme removed. The body and antrum were separated, excised into multiple slices, weighed, and then homogenized (Ultra-Turrax T25, IKA Works, Inc; 10–15 seconds at a setting of 3) in 1.5 ml sterile PBS. Antrum and body homogenates and dilutions thereof in PBS were plated for viable counts in duplicate on CBA plates containing 5-fluorocytosine (5 μ g/ml), vancomycin (10 μ g/ml), amphotericin B (5 μ g/ml), bacitracin (30 μ g/ml), polymyxin B (10 U/ml), and trimethoprim (10 μ g/ml) to suppress normal flora. Some plates also contained chloramphenicol (10 μ g/ml) for recovery of the SS1 IR203C04 strain. Plates were incubated for 5 days in an atmosphere of 5% O₂, 10% CO₂, 85% N₂. Data are presented as colony-forming units (CFU)/g antrum or body. One-way analysis of variance was used to analyze the colonization data. *p* < .05 was considered significant.

Alignments and Phylogenetic Analyses

The *hp0203*–*hp0204* intergenic region plus adjacent DNA from the corresponding -3' ends of each coding region were aligned and a consensus sequence was obtained using CLUSTALW 1.83 (<http://www.ebi.ac.uk/clustalw/>) and the aligned files were then analyzed in CLUSTALX 1.81 using the neighbor-joining method. The output file (tre format) was opened in TREEVIEW 1.6.6 (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>) and formatted into a slanted cladogram.

GenBank Accession Numbers

The sequence data for the 3' ends of the coding regions of *hp0203* and *hp0204* along with the intergenic region in between have been deposited into the GenBank database as accession numbers DQ538310–DQ538326.

Results

We developed a new complementation system for *H. pylori* using the intergenic plasmid pIR203C04 (see Fig. 1; Materials and Methods) and used the *H. pylori* arginase gene as a model to validate this system. After the wild-type or arginase mutant 26695 *H. pylori* derivatives were transformed with the intergenic plasmid derivatives, transformants were confirmed by PCR analysis and tested for phenotypic effects by arginase activity, Western blot, and L-arginine consumption. Furthermore, transformation frequency was examined in 26695, as well as SS1, J99, and 43504 *H. pylori* strains to determine the application of this complementation method to genetically diverse strains. Finally, the usefulness of the complementation system was examined in vivo using a mouse model.

Confirmation of *H. pylori* Strains by PCR and Southern Blot

All genetically engineered *H. pylori* strains in Table 1 were confirmed by PCR (Fig. 2A–D; data not shown). The product size when using the *cat*-F2 and *hp0203*-F1 primers was 550 bp for 26695–203C04 and 1.65 kb for the complemented strains *rocF*26695-MLB001, *rocF*26695-MLB002, *rocF*26695-MLB003, and *rocF*26695-MLB004. Wild-type 26695 and *rocF* mutant 26695 had no product with these primers, as expected. The product size using the *hp0203*-F1 and *rocF*-F25 primers was 1.15 kb for *rocF*26695-MLB001, *rocF*26695-MLB002, and *rocF*26695-MLB003. The *rocF* gene in *rocF*26695-MLB004 is in the forward orientation, so the *rocF*-R25 and *hp0203*-F1 primers were used, generating a product of 1.5 kb. Wild-type 26695, *rocF* mutant 26695, 26695–203C04, and *rocF*26695–203C04 all produced no products with these primers, as expected. The same confirmation strategy was also used for strains SS1, J99, and 43504 (data not shown). In addition, PCR was executed on all strains of *H. pylori* carrying the original *rocF*::*aphA3* mutation by using the *kan*-F2 and *rocF*-R25 primers. The product was 650 bp, demonstrating that the original *rocF* mutation was still intact; strains without the original *rocF* disruption showed no product, as expected. Southern blots were also performed using a pBS probe. The positive control plasmid (pBS) hybridized with the probe, but none of the chromosomal DNAs from the *H. pylori* strains hybridized with the pBS vector probe, confirming that the recombination did not occur as a single cross-over event (data not shown).

Complementation of Arginase Mutant *H. pylori*: Restoration of Arginase Activity

Arginase activity was determined for *E. coli* strains harboring the intergenic plasmid vectors (pMLB001–004) containing the *rocF* gene from four different *H. pylori* strains. High arginase activity occurred in *E. coli* harboring all four of these plasmids, whereas *E. coli* carrying the isogenic vector control, pIR203C04, expressed no arginase activity (Fig. 3A). Clear variation in arginase activity was conferred to the *E. coli* strains by the *rocF* genes derived from different

H. pylori strains (Fig. 3A). In the *H. pylori* strains, arginase activity determined for the wild-type 26695, 26695-203C04, and the *rocF*26695-MLB004 complemented strains were not significantly different (Fig. 3B). Also, the *rocF* mutant and *rocF*26695-203C04 strains were devoid of arginase activity, indicating that the *rocF* disruption abolishes production of arginase in these strains. The *rocF*26695-MLB001, *rocF*26695-MLB002, and *rocF*26695-MLB003 strains, which are complemented with wild-type *rocF* genes from strains 43504, SS1, and J63, respectively, all exhibited activity higher than *rocF*26695-MLB004 (Fig. 3B). Thus, even when four different *rocF* alleles were in the same genetic background (either the *E. coli* model or the *H. pylori*), arginase activity variation was evident.

In the mouse-adapted SS1 *H. pylori* strain, the *rocF* mutation also results in abolished arginase activity, whereas complementation of the arginase mutant with the *rocF* gene from SS1 (SS1 *rocF*/*rocF*⁺ in Table 1) restored arginase activity (data not shown).

Complementation of Arginase Mutant *H. pylori*: Restoration of Arginase Protein by Western Blot Analysis

Western blot analysis was performed to determine the presence of arginase protein produced in each *H. pylori* 26695 derivative, as well as DH5a *E. coli* strains carrying the plasmid derivatives. DH5a *E. coli* harboring pMLB001, pMLB002, or pMLB003 all had similar RocF levels, while *E. coli* containing pMLB004 expressed higher levels (Fig. 4A). Arginase protein observed in the wild-type 26695 versus 26695-203C04 was not significantly different, suggesting that the addition of *cat* cassette and the MCS inserted between the IR203 and IR204 regions did not alter the production of the arginase protein (Fig. 4B). The amount of arginase protein found in *rocF*26695-MLB004 was also not significantly different from the wild-type 26695, since this strain is complemented with a wild-type copy of 26695 *rocF*. Both the *rocF* mutant and the *rocF*26695-203C04 strains were devoid of arginase protein, as expected (Fig. 4B). The *rocF*26695-MLB001, *rocF*26695-MLB002, and *rocF*26695-MLB003 complemented strains exhibited also had restored expression of the arginase protein (Fig. 4B).

Complementation of Arginase Mutant *H. pylori*: Restoration of L-arginine Consumption

To provide further evidence for complementation of the arginase mutant, L-arginine consumption experiments were conducted. Since *H. pylori* cannot synthesize its own arginine [21,25–27], wild-type organisms consume L-arginine from the media in which they are grown [25–28]. *H. pylori* 26695 derivative strains were grown for 48 or 72 hours, and L-arginine consumption was measured (Fig. 5; 48-hour data shown). The *rocF* mutant strains consumed little to no L-arginine, similar to the uninoculated media; strains with measurable arginase activity (either wild type or complemented with a wild-type *rocF*) all consumed significant L-arginine in the medium. Strains grown for 72 hours that exhibited arginase activity consumed additional arginine, while strains devoid of arginase activity still did not consume significant arginine (data not shown).

Transformation Frequency in Different *H. pylori* Strains Using the Intergenic Complementation Plasmid or a Two-Step Transformation Using Chromosomal DNA

To determine whether the complementation system could be used for other *H. pylori* strains, transformation frequency was compared in wild-type *H. pylori* strains 26695, SS1, J99, and 43504 transformed with pIR203C04, pMLB004, 26695-203C04 chromosome, or *rocF*26695-MLB004 chromosome. Data are presented as four independent experiments (Table 3). While the intergenic plasmids transform 26695, transformation was not possible (strain SS1) or observed at low frequencies (strains 43504, J99) in the other strains studied. We therefore examined a two-step transformation, which entails transforming strain 26695 with the intergenic plasmid, isolating the resultant chromosomal DNA and then transforming this DNA into the *H. pylori* strain of interest. This two-step procedure was successful in all non-26695

strains of *H. pylori*, but had lower transformation frequencies in strain SS1 than strains 43504 and J99. With strain J99 the two-step transformation was at least two orders of magnitude more successful than transforming with the intergenic plasmids directly into J99 (Table 3). These results suggest that the complementation system may be applied to a broad range of *H. pylori* strains, including strains (43504, SS1) used in animal studies.

Variation in Intergenic Region in Different *H. pylori* Strains

To determine whether there is conservation of the *hp0203–204* intergenic region in different *H. pylori* strains, the 3' ends of the *hp0203* and *hp0204* coding regions plus the intergenic region were PCR-amplified (using primers DM71 and DM 189 [Table 2]) and directly sequenced from 17 *H. pylori* strains, including 12 minimally passaged clinical isolates. The results indicated that the 3' ends of both the *hp0203* and *hp0204* coding regions were highly conserved across the isolates (Fig. 6A,B). Strikingly, the intergenic region was not conserved in length (Table 4), and there was also substantial sequence heterogeneity (Fig. 6C,D). Remarkably, nucleotide sequences of the *hp0203–204* intergenic region were distinct from all strains except strains J75 and B194A. The length of the intergenic region largely correlated with its phylogenetic placement (Table 3 and Fig. 6D). For example, the intergenic region in strains J68, J75, B194A, 3401, and 43504 were similar in length (262–290 bp) and were clustered on the phylogenetic tree (Fig. 6D). Intriguingly, the phylogenetic trees created from the alignments of the 3' ends of *hp0203* and *hp0204* coding regions were dissimilar to each other and to the intergenic region (data not shown). The high conservation of the *hp0203* and *hp0204* 3' ends of the coding regions, however, would potentially allow use of the complementation system in a wide variety of *H. pylori* strains via the two-step transformation method.

Two other observations were made regarding the sequence data obtained on this region. First, the predicted C-terminal end of the Hp0203 protein is nine amino acids longer than reported in the original annotation of strain 26695 [21], owing to an extra G residue between nucleotides 236 and 237 of the entire coding region of *hp0203* (Fig. 6A, arrow). This one nucleotide insertion results in a shift in the reading frame that leads to a new stop codon further downstream. The sequenced strain J99 and all strains examined in this study including 26695 have this extra G residue. This updated Hp0203 protein still shares no homology in the database with any other protein, except for the J99 homolog (Jhp_0189).

Second, we noticed several interesting regions in the *hp0203–hp0204* intergenic region. The first is a repeat element, which is found in 12 of 17 strains in either one copy (strains SS1, J63 and B128A), two copies (strains B223A, B228A, J99, J104, J254, J178, and 26695 strains), or three copies (strains J188 and J166) (Fig. 6C, boxed). The consensus sequence is TTA(A/C)(T/C)A(G/C)CTATT(G/A). When the sequence is found as TTAATAGCTATTA (as in one of the copies in strains J99, J166, J178, and J188), there is a perfect palindrome (underlined). The second element we identified is TATACTGACTATTATAATAGTCGGTTATA and forms a near perfect palindrome found in strains B223A, B228A, J99, J104, J254, J188, and J166, with remnants of the element in J178 (Fig. 6C, boxed). The specific roles of these elements are unknown.

The Intergenic Complementation System Can be Used In Vivo

A major drawback to using shuttle plasmids is that antibiotic selection would likely be required for use in animal experiments, leading to potential effects on distributions and levels of normal flora organisms that could affect *H. pylori* colonization or subsequent immune response. In vitro, the intergenic complementation system did not require antibiotic selection to maintain the chromosomal construct. To ascertain whether insertions into the chromosomal *hp0203–hp0204* intergenic region affected the ability of *H. pylori* to colonize mice, the following experiment was conducted. An SS1 strain containing the *cat* cassette inserted into the intergenic

region (SS1 IR203C04 in Table 1) was constructed by the two-step transformation procedure and compared for its ability to colonize mice versus the isogenic wild-type SS1, the *rocF* SS1 mutant and the *rocF* complemented SS1 strain (see Materials and Methods and Table 1). None of the mice were given antibiotics. The results showed that all four strains of *H. pylori* colonized at similar densities to each other in both the antrum and the body ($p > .05$) (Table 5). Strains carrying antibiotic resistance prior to infection still retained proper antibiotic resistance profiles after infection (data not shown). We previously reported that the *rocF::aphA3* mutant of SS1 colonized Swiss mice at a density of about 1 log lower than wild-type mice [29]. Using a higher starting inoculum, shorter infection time, and different mouse strain than in our previous study, we observed a trend towards a 0.5 log decrease in colonization by the *rocF* mutant in both the antrum and the body. This result, however, did not reach statistical significance ($p > .05$). We conclude that the insertion of *cat* by itself or along with *rocF* into the *hp0203–204* intergenic region does not affect colonization ability, indicating that this chromosomal site can be used for in vivo complementation experiments.

Discussion

Complementation systems are important for assigning and confirming functions of specific genes and for helping to rule out polar effects. Other published methods of gene complementation within the *H. pylori* chromosome involve homologous recombination into a region critical for virulence, such as urease [12–14]. While this method has been successful in complementing *H. pylori* mutations, it disrupts the gene coding for the α -subunit of the enzyme urease, *ureA*. This presents a disadvantage when performing in vivo studies, as urease is required for colonization [30]. Another system interrupts the gene associated with metronidazole resistance, *rdxA* [15]. Growth of the *H. pylori* *rdxA* mutant with metronidazole results in altered protein profiles [31], suggesting that the *rdxA* mutation can potentially cause unwanted phenotypic effects on the cell. Whether any of these alterations affect *H. pylori* colonization or host response is unclear. Therefore, we sought to create a system for complementing mutations within a chromosomal region of *H. pylori* that is likely unassociated with pathogenicity and with minimal chances of pleiotropic and polar effects. The plasmid base, pBluescript, was selected for use because native *E. coli* plasmids are neither replicated nor stably maintained within *H. pylori*. The intergenic region within the complementation plasmid pIR203C04 undergoes homologous recombination into the *H. pylori* *hp0203–204* intergenic region. This serves as an attractive alternative to the more traditional shuttle plasmid-mediated gene complementation systems. The system developed here also eliminates copy number effects from shuttle plasmids.

The site chosen for the chromosomal complementation system described here was based on the finding that the *hp0203–204* intergenic region is one of the largest in the genome and seemed to be reasonably well conserved based on strains 26695 and J99 [20,21]. The *hp0203* and *hp0204* coding regions are transcribed in opposite directions towards each other (Fig. 1A). This setting minimizes the possibility of creating polar effects after recombination has occurred, and minimizes risk of inserting DNA fragments into a promoter region. The striking variability in the *hp0203–204* intergenic regions across different *H. pylori* strains, however, suggests that an improved derivative of the system will be required, in which the flanking, highly conserved *hp0203* and *hp0204* coding regions are utilized. With this proposed modification, the complementation would still be targeted to the *hp0203–204* intergenic region. This system is currently under development.

Using the pIR203C04-based chromosomal-targeting complementation plasmid, homologous recombination events occurred not only in strain 26695, but also in 43504 and J99. This occurred despite existence of only ~330 bp of flanking *H. pylori* DNA in the intergenic plasmid. In contrast, strain SS1 required a two-step transformation. The reason for this is unclear since

the SS1 *hp0203–204* intergenic region is phylogenetically closer to the 26695 region than strain 43504 is to 26695 (Fig. 6C,D). We speculate that SS1 is more recalcitrant to transformation due to more abundant or distinct restriction–modification systems than found in other strains. Variation in transformation frequencies, when 26695-derived DNA was transformed into heterologous strains, was also probably due to differences in restriction–modification systems, which have been suggested to be important contributors to strain-dependent variations in transformation frequencies [20,21,32]. The variability of the *hp0203–204* intergenic region itself could also play a role in transformation frequency differences. To test this idea, the intergenic plasmids derived from different *H. pylori* strains could be analyzed for transformation frequencies in a single *H. pylori* strain background.

While the intergenic plasmids were unable to transform strain SS1, transformation using chromosomal DNA from the recombinant 26695-203C04 and *rocF*26695-MLB004 (via two-step transformation) was achievable in all three heterologous strains studied, suggesting that this complementation system can be adapted to numerous strains. Presumably, the high degree of conservation of the *hp0203* and *hp0204* coding regions allowed for these recombination events, since all the recombinant strains were shown to be correct by PCR analysis. We recognize that the two-step transformation procedure, while potentially allowing use of the complementation system in broad *H. pylori* strain backgrounds, has the limitation of generating possible recombination events far away from the *hp0203* and *hp0204* coding regions, leading to strains that are not truly isogenic.

The complementation system was applied to the arginase mutant of *H. pylori*. While we cannot completely rule out the possibility for unknown secondary effects in the complemented strains, phenotypes lost in the *rocF* mutant (arginase activity, arginase protein, arginine consumption) were all restored in the complemented strain. Interestingly, arginase genes from distinct *H. pylori* strains confer variable arginase activity in the same *H. pylori* genetic background. For example, the data in Fig. 3B suggest that the *rocF* genes from strains SS1, 43504, and J63 confer higher arginase activity than *rocF* from strain 26695, when all *rocF* genes are in the 26695 *rocF* mutant strain background. We hypothesize that the sequence variation in the arginase coding region or upstream region plays an important role in the amount of arginase activity observed. This hypothesis is being studied elsewhere (Hovey and McGee, manuscript in preparation). When the 26695 *rocF* mutant was complemented with a wild-type copy of *rocF* from 26695 (*rocF*26695-MLB004), arginase protein and activity were not significantly different from the original wild-type 26695, thereby producing a true complement of the arginase mutant.

Arginase has been shown to be regulated post-translationally by thioredoxin-1 [33], but nothing is yet known about sequence variation of thioredoxin-1 in different *H. pylori* strains. Recent evidence also suggests that arginase is transcriptionally regulated by the ArsR/S (Hp0165/0166) two-component signal transduction system [34].

Significant genetic polymorphisms exist between different strains of *H. pylori* [35] (Fig. 6 and Table 4), and horizontal gene transfer is an important contributor to such diversity [36,37]. The high degree of heterogeneity in the *hp0203–hp0204* intergenic region suggests there is low selective pressure to maintain conservation of this region. This intergenic region does not appear to play an important role for *H. pylori* in vivo. Supporting this idea is our finding that insertion of a *cat* cassette alone or in conjunction with the wild-type arginase gene (*rocF*) does not alter the ability of *H. pylori* SS1 to colonize mice (Table 5). Whether the flanking coding regions (*hp0203* and *hp0204*) are important for colonization is unknown, but these regions have a high degree of conservation (Fig. 6A,B), suggesting they have a function that necessitates maintenance of selective pressure on these coding regions. The finding that 16 of

17 strains had distinct *hp0203–204* intergenic sequences in our small scale analysis raises the intriguing possibility that this region could be utilized as a novel typing method.

This gene complementation system is a valuable tool that will provide ability to study genes in the native *H. pylori* background, to investigate the microheterogeneity of *H. pylori* genes all in the same genetic background, to allow overexpression of genes under the control of a strong promoter, to allow in vivo complementation experiments in the absence of antibiotic selection and to minimize polar and pleiotropic effects. This complementation system should greatly facilitate genetics experiments in *H. pylori*.

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Figure 1.

Development of an *H. pylori* chromosomal complementation system. (A) Wild-type 26695 *H. pylori* chromosome region chosen for complementation. The intergenic region (IR) was divided into two fragments for cloning purposes, IR203 and IR204. Arrow indicates direction of transcription for the coding regions. The 326 bp IR203 and 332 bp IR204 regions were cloned. The actual intergenic region size in strain 26695 is shorter (632 bp), based on sequence analysis (see Fig. 6 and Table 4). (B) Construction of cloning vectors: pIR20304, pIR203C04, pMLB001, pMLB002, pMLB003, and pMLB004. Arrows in plasmids denote direction of transcription. MCS, multicloning site.

Figure 2.

PCR confirmation of complementation strains. (A) 26695–203C04 chromosome and primers used in PCR confirmation. Expected product size using *hp0203*-F1 and *cat*-F2 primers is ~550 bp. (B) *rocF* 26695-MLB001 chromosome and primers used for confirmation. Product size between *hp0203*-F1 and *rocF*-F25 is ~1.15 kb, *hp0203*-F1 and *cat*-F2 product size is ~1.65 kb. This strain also harbors the *rocF::aphA3* mutation described in Fig. 2C. (C) *rocF* mutant (*rocF::aphA3*) 26695 chromosome, interrupted by a kanamycin cassette. Expected product size is ~650 bp using primers *kan*-F2 and *rocF*-R25. *rocF'* and *'rocF* denote incomplete open-reading frame. (D) PCR confirmation of 26695 derivative strains, using *hp0203*-F1 and *cat*-F2 primers. Black arrows denote primers. MCS, multicloning site.

Figure 3.

Arginase activity of *E. coli* expressing *H. pylori rocF* and of various *H. pylori* strains. (A) Arginase activity in *E. coli* carrying pIR203C04 (negative control), pMLB001 (43504 *rocF* gene), pMLB002 (SS1 *rocF* gene), pMLB003 (J63 *rocF* gene), or pMLB004 (26695 *rocF* gene). (B) Arginase activity in *rocF/rocF⁺* 26695 complemented strains, compared to wild-type and *rocF* mutant 26695. Strains listed in Table 1. L-orn, L-ornithine; prot, protein.

Figure 4.

Arginase production assessed by Western blot analysis. (A) Anti-RocF Western blot analysis of *E. coli* containing pIR203C04, pMLB001, pMLB002, pMLB003, and pMLB004. Protein (20 µg) was added per well. (B) Anti-RocF Western blot analysis of *H. pylori*. Protein (20 µg) was added per well. The experiment was repeated twice (independently prepared extracts) with similar results.

Figure 5.

L-arginine consumption by wild-type and genetically engineered *H. pylori* strains. *H. pylori* 26695 strains were inoculated at 5×10^5 CFU/ml in RPMI 1640 medium containing 10% FBS and grown for 48 hours. The culture supernatant was measured for L-arginine by HPLC-ECD as described in Materials and Methods. Control, uninoculated media.

A

J99 AATATTACTTGCAAGACCCACAAGAGCGTGATCACAAGCTTGAAAAATGCCACAAGCCT 60
 J178 AATATTACTTGCAAGACCCACAAGAGCGGTATCACAAGCTTGAAAAATGCCACAAGCCT 60
 J254 AATATTACTTGCAAGATCCGCAAGAGCGCGATCACAAGCTTGAAAAATGCCACAAGCCT 60
 J188 AATATTACTTGCAAGATCCGCAAGAGCGCGATCACAAGCTTGAAAAATGCCACAAGCCT 60
 B223A AATATTACTTGCAAGATCCGCAAGAGCGCGATCACAAGCTTGAAAAATGCCACAAGCCT 60
 B228A AATATTACTTGCAAGATCCGCAAGAGCGCGATCACAAGCTTGAAAAATGCCACAAGCCT 60
 J104 AATATTACTTGCAAGACCCACAAGAGCGGTATCACAAGCTTGAAAAATGCCATGAAGCCT 60
 SS1 AATATTATTGCAAGACCCGCAAGAGCGCGATCACAAGCTTGAAAAATGCCATCAAGCCT 60
 J63 AATATTACTTGCAAGACCCACAAGAGCGGTATCACAAGCTTGAAAAATGCCATCAAGCCT 60
 26695 AATATTACTTGCAAGATCCGCAAGAGCGCGATCACAAGCTTGAAAAATGCCATCAAGCCT 60
 B128A AATATTATTGCAAGATCCGCAAGAGCGCGATCACAAGCTTGAAAAATGCCATCAAGCCT 60
 J166 AATATTACTTGCAAGATCCGCAAGAGCGCGATCATAAGCTTGAAAAATGCCATCAAGCCT 60
 J75 AATATTACTTGCAAGACCCGCAAGAGCGCGATCACAAGCTTGAAAAATGCCATCAAGCCT 60
 B194A AATATTACTTGCAAGACCCGCAAGAGCGGTATCACAAGCTTGAAAAATGCCATCAAGCCT 60
 J68 AATATTACTTGCAAGACCCGCAAGAGCGCGATCACAAGCTTGAAAAATGCCATCAAGCCT 60
 3401 AATATTACTTGCAAGATCCGCAAGAGCGGTATCACAAGCTTGAAAAATGCCATGAAGCCT 60
 43504 AATATTACTTGCAAGATCCGCAAGAGCGGTATCACAAGCTTGAAAAATGCCACAAGCCT 60

J99 TTGACTCGACGCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 J178 TTGACTCGACGCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 J254 TTGACTCGACGCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 J188 TTGATTGACGCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 B223A TTGATTGATGCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 B228A TTGATTGATGCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 J104 TTGATTGACGCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 SS1 TTGATTGACGCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 J63 TTGATTGACCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 26695 TTGATTGACCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 B128A TTGATTGACGCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 J166 TTGATTGACCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 J75 TTGATTGACCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 B194A TTGATTGACCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 J68 TTGATTGACCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 3401 TTGATTGACCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120
 43504 TTGATTGACGCTTAAGGTTAATTTTATCAGGAAGTCTTTTAAAAAGGATTGCAAGCATG 120

↑

J99 CGCAATGGCTTTAGAGCAAGCTCAAAAAGAACTCCATGA 161
 J178 CGCAATGGCTTTAGAGCAAGCTCAAAAAGAACTCCATGA 161
 J254 CGCAATGGCTTTAGAGCAAGCTCAAAAAGAACTCCATAA 161
 J188 CGCAATGGCTTTAGAGCAAGCTCAAAAAGAACTCCATGA 161
 B223A CACAAATGGCTTTAGAGCAAGCTCAAAAAGAACTCCATGA 161
 B228A CACAAATGGCTTTAGAGCAAGCTCAAAAAGAACTCCATGA 161
 J104 CGCAATGGCTTTAGAGCAAGCTCAAAAAGAACTCCATGA 161
 SS1 CGCAATGGCTTTAGAGCAAGCTCAAAAAGGGAAGTCCATAA 161
 J63 CGCAATGGCTTTAGAGCAAGCTCAAAAAGGGAAGTCCATAA 161
 26695 CGCAATGGCTTTAGAGCAAGCTCAAAAAGGGAAGTCCATAA 161
 B128A CACAAATGGCTTTAGAGCAAGCTCAAAAAGGGAAGTCCATAA 161
 J166 CGCAATGGCTTTAGAGCAAGCTCAAAAAGAACTCCATGA 161
 J75 CGCAATGGCTTTAGAGCAAGCTCAAAAAGGGAAGTCCATGA 161
 B194A CGCAATGGCTTTAGAGCAAGCTCAAAAAGGGAAGTCCATGA 161
 J68 CGCAATGGCTTTAGAGCAAGCTCAAAAAGGGAAGTCCATGA 161
 3401 CGCAATGGCTTTAGAGCAAGTCCCAACAAAAGGCTCCATGA 161
 43504 CTCAATGGCTTTAGAGCAAGTCCCAACAAAAGGCTCCATGA 161

C

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SS1 -----AAGGGGTTCTTTAGGGATTTTATTTTC-TTATAGCAGAA 38
J63 -----AAGGGGTTCTTTAGGGATTTTATTTTC-TTATAGCAGAA 38
B223A -----AGGGGTTCTTTAGGAATTTTATTTT-CTATAGCAGAA 37
B228A -----AGGGGTTCTTTAGGAATTTTATTTT-CTATAGCAGAA 37
J99 -----AGGGGTTCTTTAGGAATTTTATTTTC-CTATAGCAGAA 37
J104 -----AGGGGTTCTTTAGGAATTTTATTTTC-CTATAGCAGAA 37
J254 -----AGGGGTTCTTTAGGAATTTTATTTTC-CTATAGCAGAA 37
J188 -----AGGGGTTCTTTAGGAATTTTATTTTC-TTATAACAGAA 37
J166 -----AGGGGTTCTTTAGGAATTTTATTTTC-CTATAGCAGAA 37
J178 -----AGGGGTTCTTTAGGAATTTTATTTTC-CTATAGCAGAA 37
26695 -----AAGGGGTTCTTTAGGGATTTTATTTTC-TTATAGCAGAA 38
B128A -----AAGGGGTTCTTTAGGGATTTTATTTTC-TTATAGCAGAA 37
J75 GAGGTTTTGTCGCTTTGTGGCAAACTCTTTTT-AAATTTTATCCCACTAGCACAAA 58
B194A GAGGTTTTGTCGCTTTGTGGCAAACTCTTTTT-AAATTTTATCCCACTAGCACAAA 58
J68 GAGGTTTTGTCGCTTTGTGGCAAACTCTTTTT-AAATTTTATCCCACTAGCACAAA 58
43504 GAGGTTTTGTCGCTTTGTGGCAAACTCTTTTTTAAATTTTATCCCACTAGTGCGAAA 60
3401 GAG---TGGTCGCTTTGTGGCAAACTCTTTTT-AAATTTTATCCCACTAGTGCAAAA 55
          * * * * *

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Repeat

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SS1 ATTATT---TTTAAAGCAAAGGCA-AATCATGTTTGTATTAATTAGAGATATATTGTT 93
J63 ATTATT---TTTAAAGTAAAAGACA-AATCATGTTTGTATTAATTAGAGATATATTGTT 93
B223A ATTATT---TTTAAAGTAAAAGACG-AATAATGTTT-----AAAGGCATTCTAGT 83
B228A ATTATT---TTTAAAGTAAAAGACG-AATAATGTTT-----AAAGGCATTCTAGT 83
J99 ATTATT---TTTAAAGTAAAAGACG-AATAATGTTT-----AAAGGCATTCTAGT 83
J104 ATTATT---TTTAAAGTAAAAGACG-AATAATGTTT-----AAAGGCATTCTAGT 83
J254 ATTATT---TTTAAAGTAAAAGACG-AATAATGTTT-----AAAGGCATTCTAGT 83
J188 ATTATT---TTTAAAGTAAAAGACG-AATAATGTTT-----AAAGGCATTCTAGT 83
J166 ATTATT---TTTAAAGTAAAAGACG-AGTAATGTTT-----AAAGGCATTCTAGT 83
J178 ATTATT---TTTAAAGTAAAAGATG-AATAATGTTT-----AAAGGCATTCTAGT 83
26695 ATTATT---TTTAAAGTAAAAGACA-AATCATGTTT-----AGAGATATAGTAGA 84
B128A ATTATT---TTTAAAGCAAAGACA-AATTATGTTTGTATTAATTAGAGATATAGTAAT 92
J75 AACTCTGTCCTTTAGGGTGCAGATGTAAGCACATAGCCGTTAGGCTAAAAGAGGTTA-A 117
B194A AACTCTGTCCTTTAGGGTGCAGATGTAAGCACATAGCCGTTAGGCTAAAAGAGGTTA-A 117
J68 AACTCTGTCCTTTAGGGTGCAGATGTAAGCACATAGCCGTTAGGCTAAAAGAGGTTA-A 117
43504 AACTCTGTCCTTTAGGGTGCAGATGTAAGGCATAGCCGTTAGGCTAAAAGAGGTTA-A 119
3401 AACTCTGTCCTTTAGTTCGCAAGATGCAAGCACATAGCCGTTAGGCTAAAATAGGTTGA 115
          * * * * *

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Repeat

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SS1 TATTCTACTACCTATTATGGCTATTGGA-TTTGTATTAGCGCT----- 136
J63 TATTCTACTACCTATTATGGCTATTGGA-TTTGTATTAGCAGCT----- 136
B223A TTTTCTAATACCTATTGTTCTTATTGGG-TTAGTATTAA----- 121
B228A TTTTCTAATACCTATTGTTCTTATTGGG-TTAGTATTAA----- 121
J99 TTTTCTAATACCTATTGTTCTTATTGGG-TTAGTATTAA----- 121
J104 TTTTCTAATACCTATTGTTCTTATTGGG-TTAGTATTAA----- 121
J254 TTTTCTAATACCTATTGTTCTTATTGGG-TTAGTATTAA----- 121
J188 TTTTCTAATACCTATTGTTCTTATTGGG-TTAGTATTAAATAGCTATTATTATT--GGGT 139
J166 TTTTCTAATACCTATTGTTCTTATTGGGGTTAGTATTAAATAGCTATTATTATTATTGGGT 143
J178 TTTTCTAATACCTATTGTTCTTATTGGG-TTAGTATTAA----- 120
26695 TATTCTAATACCTATTGTTCTTATTGGG-TTAGTATTAA----- 122
B128A TATTCTACTACCTATTATGAACCAAGTAA--AGTATGCT----- 128
J75 AATACCCTAGTCTTTT-TAGGGTAGGA--AATATCCATTGACA----- 158
B194A AATACCCTAGTCTTTT-TAGGGTAGGA--AATATCCATTGACA----- 158
J68 AATACCCTAGTCTTTT-TAGGGTAGGA--AATATCCATTGACA----- 158
43504 AATACCCTAGTCTTTT-TAGGGTAGGA--AATATCCATGACA----- 160
3401 AATACCCTAGTCTTTTATAGGGTAGGA--AATGTCATGACA----- 157
          * * * * *

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Repeat

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SS1 -----TGTGAGAGGTAGAG--ATGGTGATG-ACGAT-AATGATGTTAATGATGGT-- 183
J63 -----TGTGAGAGGTAGAG--ATGGTGATG-ACGAT-AATGATGTTAATGATGGT-- 183
B223A -----CAGCTATTAGAGCTACTATAATG-GCGTTTAGAGGCGATACTGATGGT-- 168
B228A -----CAGCTATTAGAGCTACTATAATG-GCGTTTAGAGGCGATACTGATGGT-- 168
J99 -----TAGCTATTAGAGCTACTATAATG-GCATTTAGAGGCGATACTGATGGT-- 168
J104 -----CAGCTATTAGAGCTACTATAATG-GCATTTAGAGGCGATACTGATGGT-- 168
J254 -----CAGCTATTAGAGCTACTATAATG-GCATTTAGAGGCGATACTGATGGT-- 168
J188 -----TAGCTATTAGAGCTACTATAATG-GCATTTAGAGGCGATACTGATGGT-- 195
J166 TAGTATTAAACAGGTATTAGAGCTACTATGATG-GTGTTAGAGGCGATACTGATGGT-- 199
J178 -----TAGCTATTAGAGCTACTATAATG-GCATTTAGAGGCGATACTGATGGT-- 167
26695 -----CAGCTATTAGAGCTACTATAATG-GCGTTTAAAGGCGATACTGATGATGAT 172
B128A -----TAAATTTAATTTTATTAGTCTGTTTTTAAAGTCAATGCTTGGCTA 176
J75 -----CTTTAAAGAACAAAGCCTTTCTGTTAGCATTAAATGGAATGCTT--TAGTTA 209
B194A -----CTTTAAAGAACAAAGCCTTTCTGTTAGCATTAAATGGAATGCTT--TAGTTA 209
J68 -----ATTTAAAGAACAAAGCCTTTCTGTTAGCATTAAATGGAATGCTT--TAGTTA 209
43504 -----CTTTAAAGAACAAAGCCTTTCTGTTAGCATTCAATGGAATGCTT--TAGTTA 211
3401 -----CTTTAAAGAACAAAGTCTTTCTGTTAGCATTCAATGGAATGCTT--TAGTTA 208
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Figure 6.

Variation in the *hp0203*–*hp0204* intergenic region across *H. pylori* strains. The 3' ends of the *hp0203* and *hp0204* coding regions, plus the intergenic region was PCR-amplified and sequenced from 17 *H. pylori* strains. (A) Multisequence nucleotide alignment of the 3' end of *hp0203* coding region using clustalw. Arrow, extra residue uncovered in the analysis that alters the Hp0203 C-terminal region (lowercase nucleotide). Asterisks depict residues conserved in all 17 *H. pylori* strains. Underlined, stop codon for *hp0203*. (B) Multisequence nucleotide alignment of the 3' end of *hp0204* coding region using clustalw. Underlined, stop codon for *hp0204*. (C) Multisequence nucleotide alignment of the *hp0203*–204 intergenic region using clustalw. Boxed, repeat elements and palindromes. Underlined, possible stem-loop structures for *rho*-independent transcriptional termination as determined by m-fold. (D) Bootstrap neighbor-joining phylogenetic tree (slanted cladogram) of the alignment in Fig. 6C using clustalx and treeview. Asterisk denotes that these strains are 100% identical at the nucleotide level over the entire intergenic region.

Table 1

Description of strains used in this study

Bacterial Strain	Description	Source
<i>H. pylori</i>		
26695	Wild-type <i>H. pylori</i> strain	[21]
26695 <i>rocF::aphA3</i> (26695 <i>rocF</i> mutant)	Contains <i>rocF</i> gene disrupted by kanamycin (<i>aphA3</i>) cassette	[29]
26695-203C04 (IR203:: <i>cat</i> 204)	26695 transformed with pIR203C04 (chloramphenicol resistance <i>cat</i> cassette inserted into <i>hp0203–204</i> intergenic region)	This study
<i>rocF</i> 26695-203C04 (IR203:: <i>cat</i> 204)	26695 <i>rocF</i> mutant transformed with pIR203C04 (chloramphenicol resistance <i>cat</i> cassette inserted into <i>hp0203–204</i> intergenic region)	This study
<i>rocF</i> 26695-MLB001 (IR203:: <i>cat rocF</i> 204)	26695 <i>rocF</i> mutant transformed with pMLB001 (<i>rocF/rocF</i> ⁺)	This study
<i>rocF</i> 26695-MLB002 (IR203:: <i>cat rocF</i> 204)	26695 <i>rocF</i> mutant transformed with pMLB002 (<i>rocF/rocF</i> ⁺)	This study
<i>rocF</i> 26695-MLB003 (IR203:: <i>cat rocF</i> 204)	26695 <i>rocF</i> mutant transformed with pMLB003 (<i>rocF/rocF</i> ⁺)	This study
<i>rocF</i> 26695-MLB004 (IR203:: <i>cat rocF</i> 204)	26695 <i>rocF</i> mutant transformed with pMLB004 (<i>rocF/rocF</i> ⁺)	This study
SS1	Wild-type mouse-adapted <i>H. pylori</i> strain	[38]
SS1 <i>rocF::aphA3</i> (SS1 <i>rocF</i> mutant)	Contains <i>rocF</i> gene disrupted by kanamycin (<i>aphA3</i>) cassette	[29]
SS1 IR203C04	SS1 transformed with chromosomal DNA from 26695-203C04 (chloramphenicol resistance <i>cat</i> cassette inserted into <i>hp0203–204</i> intergenic region)	This study
SS1 <i>rocF/rocF</i> ⁺ (IR203:: <i>cat rocF</i> 204)	SS1 <i>rocF::aphA3</i> transformed with chromosomal DNA from <i>rocF</i> 26695-MLB002 (WT SS1 <i>rocF</i> gene inserted into <i>hp0203–204</i> intergenic region)	This study
43504	Wild-type <i>H. pylori</i> strain	ATCC
3401	Wild-type <i>H. pylori</i> strain	M. Blaser
J63	Wild-type <i>H. pylori</i> low passage clinical isolate	R. Peek
J68	Wild-type <i>H. pylori</i> low passage clinical isolate	R. Peek
J75	Wild-type <i>H. pylori</i> low passage clinical isolate	R. Peek
J99	Wild-type <i>H. pylori</i> strain	[20]
J104	Wild-type <i>H. pylori</i> low passage clinical isolate	R. Peek
J166	Wild-type <i>H. pylori</i> low passage clinical isolate	R. Peek
J178	Wild-type <i>H. pylori</i> low passage clinical isolate	R. Peek
J188	Wild-type <i>H. pylori</i> low passage clinical isolate	R. Peek
J254	Wild-type <i>H. pylori</i> low passage clinical isolate	R. Peek
B128A	Wild-type <i>H. pylori</i> low passage clinical isolate	R. Peek
B194A	Wild-type <i>H. pylori</i> low passage clinical isolate	R. Peek
B223A	Wild-type <i>H. pylori</i> low passage clinical isolate	R. Peek
B228A	Wild-type <i>H. pylori</i> low passage clinical isolate	R. Peek
<i>E. coli</i>		
DH5α	F [−] , supE44 ΔlacU169 φ80lacZM15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1	BD Biosciences Clontech

ATCC, American Type Culture Collection.

Table 2

Description of plasmids and primers used in this study

Plasmid or primer	Description	Source
Plasmid		
pBluescript II SK(−) (pBS)	Cloning vector, ampicillin ^r (3 kb)	Stratagene
pIR204	pBS containing <i>H. pylori</i> IR204 region (3.33 kb)	This study
pIR20304	pIR204 with <i>H. pylori</i> IR203 inserted (3.66 kb)	This study
pIR203C04	pIR20304 with chloramphenicol resistance cassette (<i>cat</i>) inserted (4.5 kb)	This study
pIR203K04	pIR20304 with kanamycin resistance cassette (<i>aphA3</i>) inserted (4.9 kb)	This study
pMLB001	pIR203C04 with <i>H. pylori</i> strain 43504 <i>rocF</i> gene and native promoter inserted (5.6 kb)	This study
pMLB002	pIR203C04 with <i>H. pylori</i> strain SS1 <i>rocF</i> gene and native promoter inserted (5.6 kb)	This study
pMLB003	pIR203C04 with <i>H. pylori</i> strain J63 <i>rocF</i> gene and native promoter inserted (5.6 kb)	This study
pMLB004	pIR203C04 with <i>H. pylori</i> strain 26695 <i>rocF</i> gene and native promoter inserted (5.6 kb)	This study
pJH43504	pBluescript SK containing <i>H. pylori</i> 43504 <i>rocF</i> gene and native promoter (4.1 kb)	Hovey & McGee, unpublished
pJHSS1	pBluescript SK containing <i>H. pylori</i> SS1 <i>rocF</i> gene and native promoter (4.1 kb)	Hovey & McGee, unpublished
pJH63	pBluescript SK containing <i>H. pylori</i> J63 <i>rocF</i> gene and native promoter (4.1 kb)	Hovey & McGee, unpublished
pBS- <i>rocF</i>	pBluescript SK containing <i>H. pylori</i> 26695 <i>rocF</i> gene and native promoter (4.1 kb)	[29]
pBS- <i>cat</i>	pBS containing 0.8 kb non-polar chloramphenicol acetyltransferase cassette from <i>Campylobacter coli</i> ; subcloned from pSKCAT4 into <i>EcoRI</i> site, chloramphenicol ^r , ampicillin ^r (3.8 kb)	This study
pBS-kan	pBS containing 1.25 kb non-polar kanamycin resistance cassette (<i>aphA3</i>) from <i>Enterococcus faecalis</i> (4.3 kb)	[29]
Primers (5′–3′)		
DM3 (IR203-F1)	gcgagctcTAGAGCAAGCCCCAAAAGG	
DM4 (IR203-R1)	gctctagaTATAAAAATTAAAGCGATAA	
DM5 (IR204-F1)	ccgctcgagCGTTTTTTTGAAGCCAATTCT	
DM6 (IR204-R1)	ggggtaccTAAAATTTGGTTTTTGTTC	
DM71 (<i>hp0203</i> -F1)	CCCTCCTATGGGGTTTCATT	
DM189 (<i>hp0204</i> -F2)	CCGGTGGTGATTAAAAACCTT	
DM228 (<i>hp0204</i> -F3)	CAATAGGGGAAATTCATGCG	
<i>cat</i> -F2	AATCCCAGTTTGTCGCAC	
<i>cat</i> -F4	TGCTGCCTTGCTACACAGTT	
kan-F2	GCGCGAGCTGTATGATTTT	
<i>rocF</i> -F25	cgggatccCATGATTTTAGTAGGATTAGAAGCAGAGT	
<i>rocF</i> -R25	ggggtaccTCAGTAACTCCTTGCAAAAGAGTGCTTC	

^r, resistance.

Transformation frequency of four wild-type *H. pylori* strains: 26695, SS1, J99, and 43504 using intergenic plasmids versus chromosomal DNA derived from strain 26695 already transformed with intergenic plasmids. Wild-type strains were transformed with pIR203C04, pMLB004, 26695–203C04 chromosomal (chr.) DNA, or *rocF*26695-MLB004 chromosomal DNA. Data shown are four separate experiments, with duplicates averaged from each experiment

Table 3

Strain	DNA	Frequency (Expt 1)	Frequency (Expt 2)	Frequency (Expt 3)	Frequency (Expt 4)
26695	pIR203C04	2.2×10^{-4}	5.2×10^{-6}	1.6×10^{-6}	3.8×10^{-6}
26695	26695–203C04 chr.	2.2×10^{-4}	5.2×10^{-5}	3.3×10^{-6}	3.8×10^{-6}
26695	pMLB004	4.3×10^{-6}	1.8×10^{-7}	3.6×10^{-8}	9.6×10^{-7}
26695	<i>rocF</i> 26695-MLB004 chr.	2.2×10^{-4}	5.2×10^{-5}	1.6×10^{-6}	1.9×10^{-6}
SS1	pIR203C04	$< 10^{-10}$	$< 10^{-10}$	$< 10^{-10}$	$< 10^{-10}$
SS1	26695–203C04 chr.	3.8×10^{-6}	1.2×10^{-8}	1.0×10^{-7}	6.0×10^{-9}
SS1	pMLB004	$< 10^{-10}$	$< 10^{-10}$	$< 10^{-10}$	$< 10^{-10}$
SS1	<i>rocF</i> 26695-MLB004 chr.	$< 10^{-10}$	4.5×10^{-9}	$< 10^{-10}$	$< 10^{-10}$
J99	pIR203C04	$< 10^{-10}$	1.4×10^{-8}	4.5×10^{-8}	$< 10^{-10}$
J99	26695–203C04 chr.	2.6×10^{-3}	2.1×10^{-6}	9.0×10^{-6}	3.7×10^{-7}
J99	pMLB004	$< 10^{-10}$	4.4×10^{-9}	$< 10^{-10}$	$< 10^{-10}$
J99	<i>rocF</i> 26695-MLB004 chr.	2.6×10^{-3}	2.1×10^{-6}	4.5×10^{-6}	$< 10^{-10}$
43504	pIR203C04	$< 10^{-10}$	$< 10^{-10}$	$< 10^{-10}$	$< 10^{-10}$
43504	26695–203C04 chr.	9.5×10^{-4}	8.7×10^{-8}	4.1×10^{-6}	1.2×10^{-5}
43504	pMLB004	5.9×10^{-5}	4.6×10^{-9}	8.3×10^{-7}	5.0×10^{-8}
43504	<i>rocF</i> 26695-MLB004 chr.	6.4×10^{-5}	8.7×10^{-8}	3.3×10^{-6}	7.5×10^{-7}

Table 4Variation in the length of the *hp0203*–*hp0203* intergenic region in different *H. pylori* strains

Strain	Length of intergenic region (bp)
B128A	262
B194A	288
B223A	687
B228A	681
J63	444
J68	288
J75	288
J99	689
J104	679
J166	708
J178	656
J188	748
J254	719
SS1	444
3401	287
26695	632
43504	290

Table 5

In vivo utilization of the complementation system in *H. pylori*-infected mice. Animals were inoculated with *H. pylori* strains and euthanized 1 week later. The body and antrum portions of the stomach were processed and plated for viable counts (see Materials and Methods). There is no significant difference ($p > .05$) in colonization among the four strains in either the antrum or the body

Strain (n = number of animals inoculated)	CFU/g antrum	CFU/g body
WT SS1 (n = 8)	4.1×10^3	1.0×10^4
SS1 <i>rocF::aphA3</i> (n = 7)	1.0×10^3	6.2×10^3
SS1 <i>rocF/rocF</i> ⁺ (n = 5)	6.8×10^3	2.8×10^4
SS1 IR203C04 (n = 4)	1.7×10^4	1.7×10^4