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Andrea Castillo
Eastern Washington University, acastillo@ewu.edu

Andrew J. Woodruff

Lynn E. Connolly

William E. Sause

Karen M. Ottemann

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Recombination-Based In Vivo Expression Technology Identifies *Helicobacter pylori* Genes Important for Host Colonization▽†

Andrea R. Castillo,* Andrew J. Woodruff, Lynn E. Connolly,‡
William E. Sause, and Karen M. Ottemann*

Department of Environmental Toxicology, University of California, Santa Cruz, 1156 High St., Santa Cruz, California 95064

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Here we undertook to identify colonization and gastric disease-promoting factors of the human gastric pathogen *Helicobacter pylori* as genes that were induced in response to the stomach environment. Using recombination-based in vivo expression technology (RIVET), we identified six promoters induced in the host compared to laboratory conditions. Three of these promoters, designated P*ivi10*, P*ivi66*, and P*ivi77*, regulate genes that *H. pylori* may use to interact with other microbes or the host. P*ivi10* likely regulates the *mobA*, *mobB*, and *mobD* genes, which have potential roles in horizontal gene transfer through plasmid mobilization. P*ivi66* occurs in the cytotoxin-associated gene pathogenicity island, a genomic region known to be associated with more severe disease outcomes, and likely regulates *cagZ*, *virB11*, and *virD4*. P*ivi77* likely regulates HP0289, an uncharacterized paralogue of the *vacA* cytotoxin gene. We assessed the roles of a subset of these genes in colonization by creating deletion mutants and analyzing them in single-strain and coinfection experiments. We found that a *mobABD* mutant was defective for murine host colonization and that a *cagZ* mutant outcompeted the wild-type strain in a coinfection analysis. Our work supports the conclusion that RIVET is a valuable tool for identifying *H. pylori* factors with roles in host colonization.

Helicobacter pylori is a gram-negative bacterium that colonizes the stomachs of 50% of the world's population (66). About 10% of *H. pylori* infections result in severe gastritis, gastric ulcers, gastric cancer, and mucosa-associated lymphoid tissue lymphoma (67, 86). Variability in the genetics of both the infecting *H. pylori* strains and the infected hosts likely contributes to the wide range of disease outcomes (8). The goals of this work were to identify additional *H. pylori* virulence factors that contribute to host colonization and/or to disease development and to characterize their roles in virulence.

Several virulence factors that aid *H. pylori* in colonization of the host and contribute to disease development have been identified by a variety of methods. One of these factors is the urease enzyme that *H. pylori* needs to survive in the low-pH gastric lumen as it makes its way to the gastric mucosa, which has a more neutral pH (58). The urease enzyme buffers the bacterium by converting host-produced urea into NH_3 and CO_2. *H. pylori* also requires several motility and chemotaxis genes for colonization, presumably so that it can locate and move to its preferred site of infection and remain there (27, 28, 31, 64). Autotransporters, including the VacA protein, contribute to host colonization in several ways, possibly by damaging epithelial cells and by interfering with antigen presentation (25, 57, 68, 71, 83). Other putative autotransporters encoded by *babA* and *sabA* help *H. pylori* adhere to the gastric epithelium, likely preventing bacterial shedding with epithelial cell turnover and mucus flow (40, 54). Although not required for host colonization, the *H. pylori* virulence factor NapA (*n*eutrophil *a*ctivating *p*rotein *A*) contributes to disease development, as it promotes inflammation by attracting neutrophils and monocytes to the site of infection and also stimulates the release of reactive oxygen species from leukocytes (75). Another protein found to contribute to inflammation is OipA (*o*uter *i*nflammatory *p*rotein *A*) (91). Finally, a quite well-known *H. pylori* virulence factor is the cytotoxin-associated gene (CAG) pathogenicity island and the effector CagA. The type IV secretion apparatus encoded by CAG pathogenicity island genes promotes inflammation and injection of the effectors CagA and peptidoglycan into the host epithelial cell and provokes cell dysfunction that can lead to cell transformation (35, 60, 88).

Recently, several semiglobal screens have been used to identify additional *H. pylori* genes that are required for host colonization and/or contribute to gastric disease. Two studies used in vivo screens with libraries of transposon-mutagenized *H. pylori* strains to identify genes required for *H. pylori* host colonization (6, 41). In both of these cases, the *H. pylori* strains used to infect the host were compared with strains recovered from the stomach to find genes required for viability in the host (6, 41). These studies identified some genes that the strains had in common and some unique genes. Two other studies focused on *H. pylori* genes induced in response to the host. Two of the known *H. pylori* virulence factors, UreA and NapA, were induced during infection of the host (9), supporting the conclusion that this strategy is a good strategy for identifying other virulence factors. One study used selective capture of tran-

* Corresponding author. Present address for Andrea A. Castillo: Department of Biology, Eastern Washington University, Cheney, WA 99004. Phone: (509) 359-2866. Fax: (509) 359-6867. E-mail: acastillo@mail.ewu.edu. Mailing address for Karen M. Ottemann: Department of Environmental Toxicology, University of California, Santa Cruz, 1156 High St., Santa Cruz, CA 95064. Phone: (831) 459-4780. Fax: (831) 459-3524. E-mail: ottemann@etox.ucsc.edu.

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‡ Present address: Department of Medicine, Division of Infectious Diseases, University of California, San Francisco, San Francisco, CA 94143.

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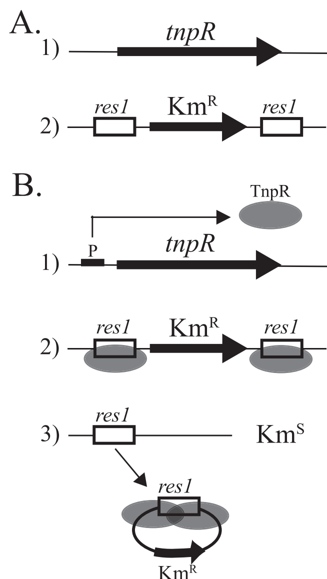

FIG. 1. RIVET for use with *H. pylori*. (A) An *H. pylori* RIVET strain contains two RIVET components located in the chromosome, a promoterless *tnpR* gene that encodes the site-specific recombinase integrated at the *H. pylori rdxA* locus (diagram 1) and a cassette in which the *aphA3* gene (also designated *Km*) that confers Km^r is cloned between *res1* sequences (*res1-Km-res1*) integrated at the HP0294-HP0295 intergenic locus (diagram 2). In the absence of an upstream promoter, *tnpR* is not expressed and the *H. pylori* strain remains Km^r. (B) In the presence of an upstream promoter (P), *tnpR* is expressed (diagram 1), and the TnpR recombinase binds the *res1* sequences to catalyze removal of the *Km* gene cloned between these sequences (diagram 2), converting the *H. pylori* strain to Km^s (diagram 3).

scribed sequence analysis to isolate *H. pylori* transcripts that were induced in human biopsies and experimentally infected gerbils and compared their expression under these conditions with the expression in culture (34). A second study used microarrays to compare *H. pylori* in vivo gene expression to expression under in vitro culture conditions (78). Although these analyses identified genes induced during host infection, each of them had its limitations. For example, the transposon mutagenesis screens did not analyze essential genes, and the transcript induction screens identified only genes that were induced at the time of RNA isolation. Thus, our goal was to identify new *H. pylori* virulence factors that may have been missed by these analyses.

Here we describe the use of recombination-based in vivo expression technology (RIVET) to identify *H. pylori* promoters induced in response to the murine host. RIVET is a variant of the original in vivo expression technology (53) in which a promoter transcriptional event is captured permanently as a conversion of the infecting strain from antibiotic resistant to antibiotic sensitive (Fig. 1) (17). The RIVET approach has been used with *Vibrio cholerae*, *Lactobacillus plantarum*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Bordetella pertussis* (14, 18, 51, 76, 87). Here we describe how we modified the RIVET system for use with *H. pylori*. Using the RIVET system, we screened a ~3,000-member library of potential *H. pylori* promoters in mice and found that 6 of them were reliably host induced. The genes regulated by these promoters include three genes with potential roles in *H. pylori* secretion systems; these genes encode Mob-like proteins potentially required for bacterial conjugation, the CagZ protein present in the CAG pathogenicity island, and the VacA paralogue encoded by HP0289. To determine whether these gene products affected animal colonization, we constructed and analyzed *mobABD* and *cagZ* gene deletion mutants. We found that the *H. pylori* Δ*mobABD* mutant was defective for host colonization, while the Δ*cagZ* mutant actually outcompeted the wild-type parent strain in competition coinfection analyses. Our work supports the conclusion that the RIVET system is a valuable tool for identifying *H. pylori* genes important for host colonization.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and antibiotics. *H. pylori* strain mG27 is a mouse-adapted descendant of clinical isolate G27 (19, 23). mG27 was generated by serially passaging the G27 *H. pylori* strain in mice (19). All *H. pylori* strains were cultured on Columbia horse blood agar (CHBA) or in brucella broth supplemented with 10% fetal bovine serum (BB10) and were grown at 37°C under microaerobic conditions with a gas mixture containing 5 to 10% O_2, 10% CO_2, and 80 to 85% N_2. Antibiotics selective for *H. pylori* were added at a concentration of either 13 μg/ml (for chloramphenicol [Cm]) or 15 μg/ml (for kanamycin [Km]). *Escherichia coli* was cultured on Luria-Bertani (LB) agar plates or in liquid media containing ampicillin (Amp) at a final concentration of 100 μg/ml. *E. coli* strains were stored at −80°C in 25 to 40% glycerol. *H. pylori* strains were stored at −80°C in brain heart infusion media supplemented with 10% fetal bovine serum, 1% (wt/vol) β-cyclodextrin, 25% glycerol, and 5% dimethyl sulfoxide.

Plasmid construction. (i) p*cat-T-tnpR*. To identify promoters that are induced during infection of animals, we created plasmid p*cat-T-tnpR*, which has a promoterless *tnpR* gene that recombines in the *H. pylori* chromosome (Fig. 2A). This plasmid contains the *Campylobacter coli* gene for Cm resistance (Cm^r) (*cat*), a strong *E. coli* transcriptional terminator ($rrnBT_1T_2$) (62), and a promoterless *tnpR* gene, all of which are flanked by sequences of the *H. pylori rdxA* gene. *cat* is transcribed from its own promoter, and the strong *E. coli* terminator $rrnBT_1T_2$, prevents read-through transcription into *tnpR* (19). The p*cat-T-tnpR* plasmid directs recombination into the middle of the *rdxA* locus on the *H. pylori* chromosome (80). *rdxA* was used because loss of this gene does not alter in vitro growth rates or the ability to infect mice (84). Upstream of the *tnpR* gene there is a unique BglII site into which the *H. pylori* genomic library was cloned.

(ii) pAW2rkr2. The *res1-Km-res1* cassette was generated by cloning the *C. coli aphA3* gene that confers Km^r (designated *Km*) between two *res1* sequences from plasmid pSL134 (82). The *res1-Km-res1* cassette was subsequently cloned into the HindIII site of pMW2 (19) in the cloned intergenic region between the convergently expressed *H. pylori* genes HP0294 and HP0295. The resulting plasmid was designated pAW2rkr2 (19). Plasmid pAW2rkr2 targets *res1-Km-res1* to the HP0294-HP0295 intergenic locus. Strain mG27 bearing this construct was designated ACHP17 (Table 1). Studies of *H. pylori* strains mG27 and SS1 containing this insertion indicated that the modification had no deleterious effects on either growth or mouse colonization (data not shown).

Generating a library of potential promoters, p*cat-T-lib-tnpR*. We generated a library of potential promoters by ligating partially Sau3A-digested genomic DNA isolated from *H. pylori* strain mG27 into the BglII site of the p*cat-T-tnpR* vector (Fig. 2A). We selected Sau3A-digested DNA fragments ranging from 1 to 4 kb long for the library by agarose gel purification. The ligated plasmids were electroporated into *E. coli* DH10B and plated onto LB medium containing Amp. Approximately 12,000 individual Amp^r colonies were pooled and grown in LB broth containing Amp, and DNA was isolated using a midiprep kit (Qiagen) to generate p*cat-T-lib-tnpR*.

Generating the *H. pylori* RIVET library. To create the *H. pylori* RIVET library, p*cat-T-lib-tnpR* was used to transform *H. pylori* strain ACHP17 (mG27 HP0294/HP0295::*res1-Km-res1*) (Table 1) to Cm^r (Fig. 2B). To minimize the number of in vitro-expressed promoter-containing clones in our *H. pylori* RIVET library, we passaged Cm^r transformants on CHBA containing Cm twice before selecting for Km^r. This step allowed in vitro-expressed clones to transcribe *tnpR*, resolve *res1-Km-res1*, and convert to Km^s (Fig. 2C). Clones that did not express *tnpR* in the lab remained Km^r and were used to generate frozen stocks for our *H. pylori* RIVET library. Independent library clones were pooled to obtain batches containing 10 clones and stored at −80°C. The *H. pylori* RIVET library consisted of ~3,000 clones.

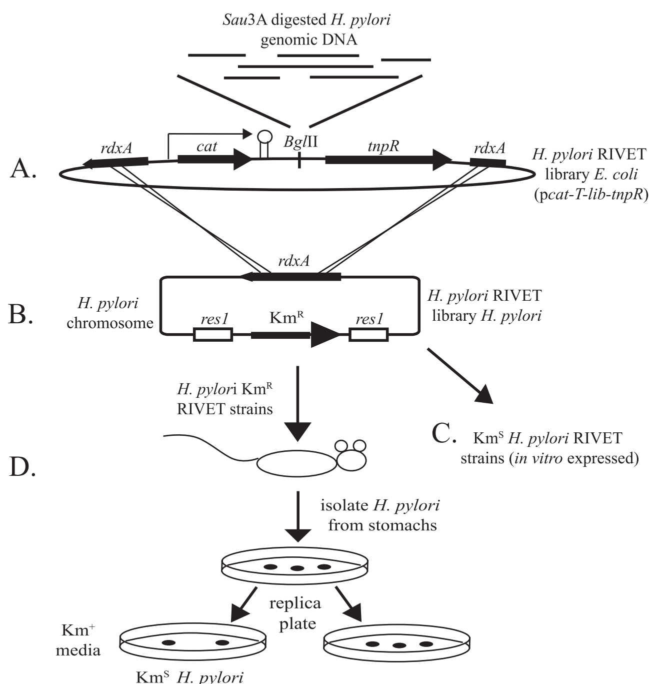

FIG. 2. Construction and screening of the p*cat-T-lib-tnpR* and *H. pylori* RIVET libraries. (A) The *H. pylori* RIVET library in *E. coli* (p*cat-T-lib-tnpR*) was generated by cloning Sau3A-digested *H. pylori* genomic DNA into the BglII site upstream of *tnpR* in plasmid p*cat-T-tnpR*. Approximately 12,000 independent clones were propagated in *E. coli*, and the average insert size was 1.0 kb. (B) The *H. pylori* RIVET library (p*cat-T-lib-tnpR*) was integrated into the *H. pylori rdxA* chromosomal locus of *H. pylori* strain ACHP17, which contained the *res1-Km-res1* cassette at an unlinked chromosomal locus. RIVET library transformants were selected on Cm-containing media. (C) In vitro expression clones were removed from the *H. pylori* RIVET library as transformants that converted to Km^s in the lab. A total of 3,340 Cm^r Km^r transformants were in our *H. pylori* RIVET library. (D) Fifty independent *H. pylori* RIVET library clones were simultaneously screened in each of two mice for in vivo induction (conversion to Km^s). After 2-weeks of infection, mouse stomachs were harvested and plated on CHBA containing Cm to isolate *H. pylori*. The plates containing CHBA supplemented with Cm were then replica plated on CHBA containing Km (Km$^+$ media) to identify in vivo-induced Km^s clones.

Analysis of p*cat-T-lib-tnpR* and the *H. pylori* RIVET library. Both p*cat-T-lib-tnpR* and the *H. pylori* RIVET library were analyzed to determine the diversity and the level of *H. pylori* genome coverage. We analyzed 30 clones obtained from the p*cat-T-lib-tnpR* library and 30 clones from the *H. pylori* RIVET library by isolating plasmid DNA and genomic DNA, respectively. The cloned *H. pylori* DNA was amplified from the templates by PCR using oligonucleotide rrnB1 or catseqst and oligonucleotide tnpRbk75 (Table 2). The presence and approximate sizes of the cloned *H. pylori* DNA fragments were determined by agarose gel electrophoresis. Cloned fragments that were similar sizes were sequenced to determine their uniqueness (Berkeley DNA Sequencing Facility, Berkeley CA). The levels of *H. pylori* genome coverage for the p*cat-T-lib-tnpR* and *H. pylori* RIVET libraries were determined using the following formula: $N = \ln(1 - P)/\ln(1 - I/G)$, where P is the probability of obtaining all clones (we used 99%), I is the insert size (we used the calculated average insert sizes, 1.0 kb for the p*cat-T-lib-tnpR* library and 0.65 kb for the *H. pylori* RIVET library), and G is the genome size (1.8×10^3 kb) (2a). Based on our calculations, the p*cat-T-lib-tnpR* library covered the *H. pylori* genome ~4.7 times and the *H. pylori* RIVET library covered 84% of the *H. pylori* genome.

Screening the *H. pylori* RIVET library for in vivo-induced promoters. All animal protocols were approved by the Institutional Animal Use and Care Committee. Fifty *H. pylori* RIVET strains were screened simultaneously for promoter induction in each of two FVB/N mice (Charles River) (Fig. 2D). Five groups of 10 *H. pylori* RIVET strains were grown on CHBA containing Km to maintain Km^r. Immediately prior to infection of mice, the 50 strains were resuspended in BB10. The bacterial concentration was determined by determining the optical density at 600 nm (OD_{600}), and the culture volume was adjusted with BB10 to obtain a bacterial concentration of $\sim 5 \times 10^7$ cells/ml. Approximately 1 ml of the RIVET strain mixture was used to infect each of two FVB/N mice that were 4 to 6 weeks old by oral gavage by using a 20-gauge, 38-mm-long needle (Popper). Infections were allowed to persist for 2 weeks, after which we harvested and processed the mouse stomachs as described by Ottemann and Lowenthal (64). In brief, the stomachs were homogenized in 500 μl BB10 using a sterile pestle, and dilutions were plated on CHBA plates. The plates were incubated for 4 days at 37°C under microaerobic conditions. *H. pylori* colonies from these plates were then replica plated on both plates containing CHBA and plates containing CHBA supplemented with Km to identify Km^s *H. pylori* RIVET strains that induced expression of *tnpR* in vivo. Km^s RIVET strains were isolated from the corresponding CHBA plates for further analysis (Fig. 2D). These strains were designated using ivi (for in vivo-induced RIVET strain) plus a number (e.g., ivi2), and the *H. pylori* DNA cloned upstream of *tnpR* in these strains were designated using P*ivi* (for in vivo-induced promoter) plus a number (e.g., P*ivi2*).

Identifying in vivo-induced promoters. To obtain the sequence representing each P*ivi* clone, we isolated genomic DNA from the Km^s *H. pylori* RIVET strains isolated from mice by using a Wizard genomic DNA purification kit (Promega). We then amplified the cloned P*ivi* region using oligonucleotides that annealed to the *cat* gene upstream of the cloned region (oligonucleotide catseqst) and the *tnpR* gene downstream of the cloned region (oligonucleotide tnpRbk75) (Table 2) by PCR. The sizes of the cloned fragments were estimated by agarose gel electrophoresis and were determined exactly by sequencing. The P*ivi* clones were sequenced using the catseqst and tnpRbk75 oligonucleotides (University of California Berkeley DNA sequencing facility). The genomic and endogenous plasmid locations of the P*ivi* clones were determined by comparing the sequences of the clones to the unpublished sequence of the *H. pylori* G27 strain genome (see Table S1 in the supplemental material). An operon analysis was done using the website http://www.microbesonline.org/.

Reconstruction of in vivo-induced strains. Since the *H. pylori* RIVET strains that were induced in the host removed the *res1-Km-res1* cassette (and were therefore Km^s), we reintroduced the *res1-Km-res1* cassette into the original locus of each of these strains before we performed our secondary analysis. One Km^s mouse output strain for each of the 13 unique Km^s ivi strains was naturally transformed with pAW2rkr2. Transformants were selected based on Km^r, and proper integration was verified by PCR using oligonucleotides that flanked the site of integration, oligonucleotides HP0294end and HP0295end (Table 2).

Testing promoter induction in vitro and in vivo. Each of the 13 reconstructed ivi strains (strains whose designations end with R in Table 1) was analyzed to determine promoter induction conferred by the P*ivi* clones in the lab and in FVB/N mice. The reconstructed ivi strains were grown on CHBA containing Km prior to these analyses to maintain Km^r. In vitro promoter induction was carried out by passaging each reconstructed ivi strain on CHBA without Km for 2 weeks (five passages on fresh CHBA). Cells of the *H. pylori* reconstructed ivi strains were then resuspended in BB10 and plated on CHBA to obtain single colonies (~200 colonies/plate). After the plates were incubated for 4 days at 37°C under microaerobic conditions, the *H. pylori* colonies were replica plated on both plates containing CHBA and plates containing CHBA supplemented with Km. The level of promoter induction (expressed as a percentage) was calculated by dividing the number of Km^s colonies by the total number of colonies and multiplying the result by 100. To determine the in vivo induction conferred by the P*ivi* regions, each reconstructed ivi strain was used independently to infect a group of FVB/N mice. After 2 weeks of infection, the mouse stomachs were harvested and plated on CHBA to isolate *H. pylori*. The plates were incubated for 4 days at 37°C under microaerobic conditions, and then colonies were replica plated on both plates containing CHBA and plates containing CHBA supplemented with Km to determine the number of *H. pylori* cells that had converted to Km^s while the strains were in mice. The level of promoter induction in vivo (expressed as a percentage) was calculated by dividing the number of Km^s colonies by the total number of colonies analyzed and multiplying the result by 100. The statistical significance of differences between promoter induction in vivo and promoter induction in vitro was calculated using the two-tailed Student *t* test.

Construction of *H. pylori* gene deletion mutants. We generated deletions of genes regulated by our in vivo-induced promoters by replacing a gene of interest with a nonpolar allele of the *C. coli cat* gene (84) that confers Cm^r. Each gene replacement cassette was generated using a PCR sewing strategy (22). In brief, chromosomal regions upstream and downstream of the gene of interest were amplified using in each case (i) one oligonucleotide that annealed to the chromosome and (ii) another oligonucleotide that annealed to the chromosome and

TABLE 1. Strains and plasmids used in this study

H. pylori strain or plasmid	Description	Reference
H. pylori strains		
mG27	Mouse-adapted G27 strain	19
SS1		47
ACHP17	mG27 HP0294/HP0295::*res1-aphA3-res1*	19
ivi2	*rdxA*::*cat-T-*P*ivi2-tnpR* HP0294/HP0295::*res1*	This study
ivi7	*rdxA*::*cat-T-*P*ivi7-tnpR* HP0294/HP0295::*res1*	This study
ivi10	*rdxA*::*cat-T-*P*ivi10-tnpR* HP0294/HP0295::*res1*	This study
ivi11	*rdxA*::*cat-T-*P*ivi11-tnpR* HP0294/HP0295::*res1*	This study
ivi23	*rdxA*::*cat-T-*P*ivi23-tnpR* HP0294/HP0295::*res1*	This study
ivi39	*rdxA*::*cat-T-*P*ivi39-tnpR* HP0294/HP0295::*res1*	This study
ivi50	*rdxA*::*cat-T-*P*ivi50-tnpR* HP0294/HP0295::*res1*	This study
ivi51	*rdxA*::*cat-T-*P*ivi51-tnpR* HP0294/HP0295::*res1*	This study
ivi63	*rdxA*::*cat-T-*P*ivi63-tnpR* HP0294/HP0295::*res1*	This study
ivi66	*rdxA*::*cat-T-*P*ivi66-tnpR* HP0294/HP0295::*res1*	This study
ivi67	*rdxA*::*cat-T-*P*ivi67-tnpR* HP0294/HP0295::*res1*	This study
ivi70	*rdxA*::*cat-T-*P*ivi70-tnpR* HP0294/HP0295::*res1*	This study
ivi77	*rdxA*::*cat-T-*P*ivi77-tnpR* HP0294/HP0295::*res1*	This study
ivi2R	*rdxA*::*cat-T-*P*ivi2-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
ivi7R	*rdxA*::*cat-T-*P*ivi7-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
ivi10R	*rdxA*::*cat-T-*P*ivi10-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
ivi11R	*rdxA*::*cat-T-*P*ivi11-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
ivi23R	*rdxA*::*cat-T-*P*ivi23-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
ivi39R	*rdxA*::*cat-T-*P*ivi39-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
ivi50R	*rdxA*::*cat-T-*P*ivi50-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
ivi51R	*rdxA*::*cat-T-*P*ivi51-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
ivi63R	*rdxA*::*cat-T-*P*ivi63-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
ivi66R	*rdxA*::*cat-T-*P*ivi66-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
ivi67R	*rdxA*::*cat-T-*P*ivi67-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
ivi70R	*rdxA*::*cat-T-*P*ivi70-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
ivi77R	*rdxA*::*cat-T-*P*ivi77-tnpR* HP0294/HP0295::*res1-aphA3-res1*	This study
Δ*mobABD*	mG27 Δ*mobABD*::*cat*	This study
Δ*cagZ*	SS1 Δ*cagZ*::*cat*	This study
Plasmids		
p*cat-T-tnpR*	*cat* gene-*E. coli rrnBT$_1$T$_2$*-BglII-*tnpR*	19
pAW2rkr2	*res1-aphA3-res1* cassette at HindIII site of pMW2	19

either the start or end of the *cat* gene. A third PCR product representing the nonpolar *cat* allele was generated using oligonucleotides catR2 and catF (Table 2). The PCR products representing the upstream chromosomal region, the downstream chromosomal region, and the *cat* gene were generated independently, purified using an agarose gel (GFX PCR DNA and gel band purification kit; GE Healthcare), and then combined. The mixture of PCR products was used as a template with the oligonucleotides that annealed to the far upstream and downstream PCR product regions. The large PCR products generated in these reactions (upstream region-*cat*-downstream region) were purified using an agarose gel and used to naturally transform *H. pylori* strains SS1 (47) and mG27 (19) to Cm^r as previously described (72). All mutant strains were found to be wild type for motility by microscopic inspection and to be wild type for urease activity using a pH indicator buffer (Difco urea broth; Difco) (data not shown).

Mouse colonization analyses. *H. pylori* strains used for colonization analyses were passaged minimally in the lab on CHBA (two or three times) and then removed either from CHBA after growth for ~18 h or from a BB10 culture grown for ~18 h. *H. pylori* strains grown on CHBA were transferred to BB10 prior to infection and analyzed to determine motility and the bacterial cell concentration (OD_{600}). *H. pylori* strains grown in BB10 were analyzed directly to determine motility and the bacterial cell concentration (OD_{600}). Approximately 1 ml of an *H. pylori* culture containing 5×10^7 to 5×10^8 CFU/ml was used to inoculate mice by oral gavage. For single-infection studies, either a mutant *H. pylori* strain or the appropriate wild-type control strain was used to infect mice. For the coinfection analysis, the mutant and wild-type strains were grown separately and analyzed to determine their motilities and bacterial cell concentrations (OD_{600}) before the cultures were mixed and used for coinfection. The bacterial cell concentrations were used to generate a mixed culture containing approximately equal numbers of cells of the mutant and wild-type strains. The actual bacterial cell concentration was determined more accurately by culture dilution and plating. Infections were allowed to persist for 2 weeks, after which the mouse stomachs were isolated and plated on CHBA as described above. The stomachs of mice infected with the mutant strains were plated on CHBA containing Cm, and the stomachs of mice infected with the wild-type strain were plated on CHBA. The stomachs of mice coinfected with both the mutant and the wild-type strain were plated on both CHBA containing Cm and CHBA. The competitive index was calculated as follows: (CFU/g for the mutant strain output/CFU/g for the wild-type strain output)/(CFU/g for the mutant strain input/CFU/g for the wild-type strain input).

RESULTS

We adapted the RIVET system used with great success by Camilli and Mekalanos (18) for use with *H. pylori* and used it to identify promoters induced in response to murine stomachs. Adapting *V. cholerae* RIVET for use with *H. pylori* required (i) creation of an *H. pylori* antibiotic resistance reporter for *tnpR* recombinase expression flanked by *res1* sites (we used Km^r [*res1-Km-res1*]) and (ii) creation of an *H. pylori* library of genomic promoters fused to the *tnpR* gene (*pcat-T-lib-tnpR*). When *tnpR* expression was directed by the cloned *H. pylori* DNA, TnpR bound the *res1* sequences and catalyzed the removal of the intervening Km^r cassette (Fig. 1).

We changed the original RIVET system from a *V. cholerae*-specific antibiotic resistance (tetracycline) system to a system

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence	Reference
catseqst	GAAGTATTATGAGGAGGGCG	19
catF2	CAACCGTGATATAGATTGAAAAGTGGAT	This study
catF	GATATAGATTGAAAAGTGGAT	This study
catR2	CGCGCCCGGGATCCTCCTTG	This study
tnpRbk75	TCAGTAAAGATGCGATTTGC	19
rrnB1	CCCTCGAGAATAAAACGAAAGGCTCAGTCG	19
G27_633_D1	GCCCTTAGTTCAGGTGTGGCAGTTTAAGG	This study
G27_633_D2	CAAGGAGGATCCCGGCCGCGGCTACCTTCTCATTTCCTAGATAGTAGCC	This study
G27_633_D3	ATCCACTTTTCAATCTATATCACGGTTGCCGGGAATGTGGGCATGCGAGTGGCG	This study
G27_633_D4	GTTTTAGCGTCAATGTTGGGGTTGATTCTAATGG	This study
G27_630_D1	GAGCTATGGGAAAGATAGAGGAAGCAATATCGC	This study
G27_630_D2	CAAGGAGGATCCCGGCCGCGCCCTCTCCTTAATTTCATACTC	This study
G27_630_D3	ATCCACTTTTCAATCTATATCACGGTTGGAACATTCTCATTTGTATGATTTGTTGAACGGG	This study
G27_630_D4	GGGCTTGAATGTCAGTGATCCTGTC	This study
G27_176_D1	GAGCGTGGATGGCAGGATCAGCGTTAAAG	This study
G27_176_D2	CAAGGAGGATCCCGGCCGCGCTCATGCATGCTTAAACCCCACATCAAGGACG	This study
G27_176_D3	ATCCACTTTTCAATCTATATCACGGTTGCGGGAGCAATCATGTTATCTTCTAATG	This study
G27_176_D4	GAATCCACGCTATAGCCTTCTTGATAC	This study
MobA_D1	CGCAATCAATCATGATAACCCTATTATATC	This study
MobA_D2	CAAGGAGGATCCCGGGCGCGCCAACATACTTGGATCTTATTTGTTC	This study
MobA_D3	ATCCACTTTTCAATCTATATCACGGTTGGAGAAGTTATAGTCGTTGGTATGGGCGGTAAG	This study
MobA_D4	CTGGTTTACTTGACATTAGATCGATAAACAGGTG	This study
HP0294end	CTTGTCCTGTGGGCGATTTGC	This study
HP0295end	GACCGGCCGGATATGGCAG	This study

that works with *H. pylori*. To do this, we chose a Km^r gene (*aphA*3 or *Km*) that carries its own promoter and flanked it with recombinase recognition sequences (*res1*) to create *res1-Km-res1*. We used the mutant *res* sequence *res1*, which contains a mutation at the crossover site resulting in decreased recombination efficiency (59). We hypothesized that use of the *res1* allele would allow us to identify promoters that were expressed to some extent in vitro but exhibited elevated expression in the host. The *res1-Km-res1* cassette was cloned into a plasmid that directed its integration into the chromosomal region between open reading frames HP0294 and HP0295 (19). We integrated this construct into the chromosome of *H. pylori* strain mG27 to ensure that a single copy was present and to ensure that it was stably maintained. *H. pylori* strain mG27 is a mouse-adapted version of the commonly used *H. pylori* strain G27 (19, 79). Integration at the HP0294-HP0295 site occurred by double-crossover gene replacement and did not affect the growth or virulence of *H. pylori* strains mG27 and SS1 (data not shown). We verified that *res1-Km-res1* integrated into the proper chromosomal region by selecting for Km^r transformants and by performing PCR with oligonucleotide primers that flanked the insertion site (data not shown). The resulting strain was designated ACHP17 (Table 1).

Generating the library of potential promoters (p*cat-T-lib-tnpR*). To generate p*cat-T-lib-tnpR* (Table 1), we created a plasmid with a promoterless *tnpR* gene and cloned a partially Sau3A-digested *H. pylori* genomic library upstream of *tnpR* (Fig. 2A). The plasmid contained the *cat* gene, which conferred Cm^r, followed by a strong *E. coli* terminator, the site for *H. pylori* library insertion, and the promoterless *tnpR* gene, all flanked by sequences of the *H. pylori* gene *rdxA*. Expression of the *cat* gene is directed by its endogenous promoter, and the *E. coli* terminator prevents read-through transcription of *tnpR* (19). We used the wild-type *tnpR* allele, which had a wild-type ribosome binding site, for our work (48). p*cat-T-lib-tnpR* was a collection of ~12,000 independent colonies. Our analysis of 30 library clones suggested that 70% of them contained inserts with an average size of 1.0 kb (data not shown). Our analysis also indicated that the library was diverse and covered the 1.8-Mb *H. pylori* genome 4.7 times (see Materials and Methods). The *rdxA* sequences flanking *cat-T-lib-tnpR* in p*cat-T-lib-tnpR* targeted integration of this plasmid into the *H. pylori* chromosomal *rdxA* locus by double-crossover homologous recombination (Fig. 2B). The *rdxA* locus is commonly used for integrating exogenous DNA into the *H. pylori* chromosome (80).

Creation of the *H. pylori* RIVET library. To construct the *H. pylori* RIVET library strains, p*cat-T-lib-tnpR* was used to transform *H. pylori* strain ACHP17 (HP0294/HP0295::*res1-Km-res1*) (Table 1) to Cm^r (Fig. 2B). Each Cm^r transformant was passaged twice in Cm-containing media before selection on Km-containing media and subsequent freezing. We initially passaged the Cm^r transformants without Km selection to allow promoters that were expressed in the lab to convert the strains carrying them to Km^s (Fig. 2C). We were interested in promoters that were not expressed in the lab or were expressed at very low levels in the lab and thus in bacteria that retained Km^r (*res1-Km-res1*). The genomic DNA of 30 Cm^r *H. pylori* transformants was screened by PCR using oligonucleotides that annealed upstream and downstream of the genomic DNA fragment to assess whether this subset of transformants contained unique genomic DNA inserts upstream of *tnpR*. The results of our screening of the *H. pylori* transformants suggested that 70% of them contained unique inserts and that the inserts were smaller (0.65 kb) than those found for the same library in *E. coli*. Although we are not certain, we speculate that the average insert size in the *H. pylori* RIVET library was smaller than the average insert size in p*cat-T-lib-tnpR* either because the larger inserts had more potential sites for *H. pylori*'s restriction systems or because larger inserts recombined less efficiently. Our *H. pylori* RIVET library contained 3,340 in-

dependent clones that were pooled in groups of 10 and stored in *H. pylori* freezing media at −80°C. These transformants covered ~84% of the *H. pylori* genome (see Materials and Methods).

Identifying *H. pylori* host-induced promoters. To identify putative host-induced promoters, we used the *H. pylori* RIVET library strains to infect FVB/N mice (Fig. 2D). We screened 50 *H. pylori* RIVET library strains in parallel by infecting mice with a mixture of 50 RIVET library strains. Baldwin and coworkers showed that this pool size allows each strain to independently establish an infection in the mouse gastric mucosa (6). The infections were allowed to persist for 2 weeks, and then we sacrificed the animals and harvested their stomachs. The stomachs were homogenized, diluted, and plated on Cm-containing media. After 4 days, each plate was replica plated on both Cm-containing media and Km-containing media (Fig. 2D). RIVET strains that induced *tnpR* expression at any time during the infection converted to Km^s. The Km^s strains were rescued from the corresponding Cm-containing plates and saved as frozen stocks for additional analysis. We screened 2,960 clones of our 3,340-clone *H. pylori* RIVET library and thus approximately 74% of the *H. pylori* genome. Our screening analysis resulted in identification of 113 Km^s *H. pylori* RIVET strains. By using PCR amplification and sequencing of the region cloned upstream of *tnpR* in these strains, we determined that the 113 Km^s strains represented 13 unique clones (see Table S1 in the supplemental material). We designated these unique clones P*ivi* clones and the strains containing them ivi strains; to identify a specific clone or strain, the number corresponding to the order of ivi strain isolation was added to its designation (Table 1).

Verifying that the *Pivi* clones were induced in the host. We retested the P*ivi* clones identified as described above to ensure that their expression in mice was greater than their expression in vitro. The ivi strains, which were isolated from mouse stomachs as Km^s strains, induced *tnpR* expression in vivo and thus removed the *res1-Km-res1* cassette from the *H. pylori* chromosome. Therefore, we reintroduced *res1-Km-res1* into these strains by transforming them to Km^r with the construct used to create the original strain (pAW2rkr2) (see Materials and Methods). Strains containing 1 of the 13 P*ivi* clones fused upstream of *tnpR* and the *res1-Km-res1* cassette (reconstructed ivi strains) were designated by adding R to the end of the ivi designation and were analyzed further to examine promoter-directed expression of *tnpR* based on their conversion from Km^r to Km^s (Table 1).

To determine P*ivi*-directed expression of *tnpR* in the host, we infected mice with each reconstructed ivi strain as a single infecting strain. Each infection was allowed to persist for 2 weeks, and then we sacrificed the animals, harvested their stomachs, and plated the stomachs to obtain single colonies on Cm-containing media as described above. Plates containing the colonies isolated from stomachs were then replica plated on Cm-containing media and Km-containing media. The level of promoter induction in vivo (expressed as a percentage) was determined by dividing the number of Km^s colonies by the total number of colonies analyzed and multiplying the result by 100. To determine the in vitro expression conferred by each P*ivi* clone, each reconstructed ivi strain was passaged on Cm-containing media in the lab for 2 weeks (four or five passages) and then plated on Cm-containing media to obtain single colonies. The resulting plates were replica plated on both Cm-containing media and Km-containing media to determine the level of promoter induction in vitro.

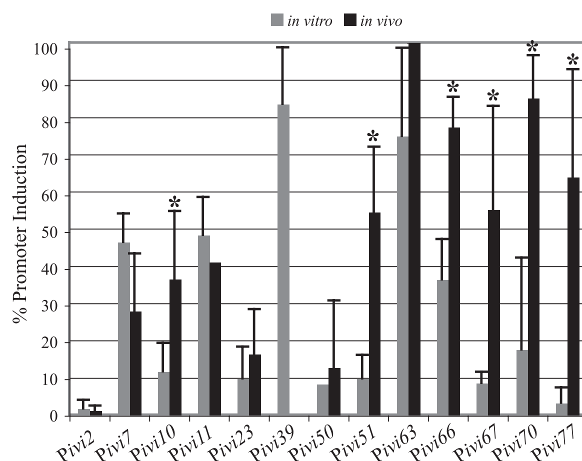

FIG. 3. Six RIVET-identified promoters were induced in the host. The relative level of promoter induction of each of the 13 reconstructed RIVET strains isolated from mice as Km^s strains was analyzed both in mice and in the lab. Promoter induction in the host was analyzed using each reconstructed RIVET strain to independently infect a group of mice for 2 weeks. Promoter induction in the lab was analyzed after 2 weeks of in vitro growth on CHBA. For both in vivo and in vitro experiments, the level of promoter induction was calculated by dividing the number of Km^s CFU isolated by the total number of CFU and multiplying the result by 100. All promoters indicated by an asterisk were induced to a greater extent in vivo than in vitro. A two-tailed Student *t* test was used to determine the following *P* values for the promoters when in vitro induction and in vivo induction were compared: for P*ivi10*, <0.1; for P*ivi51*, <0.001; for P*ivi66*, <0.001; for P*ivi67*, <0.1; for P*ivi70*, <0.05; and for P*ivi77*, <0.05. The other promoters did not differ appreciably under in vivo and in vitro conditions. The error bars indicate standard deviations. For the RIVET strains, the in vitro experiment was replicated two to six times and the in vivo analysis was carried out using zero to nine mice, as follows: for P*ivi2*, two in vitro replicates and five mice; for P*ivi7*, three in vitro replicates and five mice; for P*ivi10*, three in vitro replicates and four mice; for P*ivi11*, two in vitro replicates and one mouse; for P*ivi23*, three in vitro replicates and nine mice; for P*ivi39*, six in vitro replicates and no mice; for P*ivi50*, one in vitro replicate and two mice; for P*ivi51*, four in vitro replicates and nine mice; for P*ivi63*, two in vitro replicates and two mice; for P*ivi66*, six in vitro replicates and three mice; for P*ivi67*, two in vitro replicates and three mice; for P*ivi70*, two in vitro replicates and four mice; and for P*ivi77*, four in vitro replicates and three mice.

We found that 4 of the 13 P*ivi* clones originally isolated from mice as Km^s ivi strains conferred in vivo expression of *tnpR* that was significantly higher than the in vitro expression when the strains were tested as single infecting strains (as determined by two-tailed Student's *t* test) (Fig. 3). These P*ivi* clones were P*ivi51* ($P < 0.001$), P*ivi66* ($P < 0.001$), P*ivi70* ($P < 0.05$), and P*ivi77* ($P < 0.05$). The values for two additional P*ivi* clones, P*ivi10* and P*ivi67*, were very close to our cutoff for statistical significance, with *P* values of <0.1. We included these six P*ivi* clones in our subsequent analyses. The remaining seven P*ivi* clones identified in our screen as Km^s ivi strains did not show in vivo induction of *tnpR* when they were retested as single infecting strains (Fig. 3). It is possible that the P*ivi* clones either were simply not induced in mice and were isolated as background clones inherent in this screen or were induced only when the strains were used in coinfections with additional

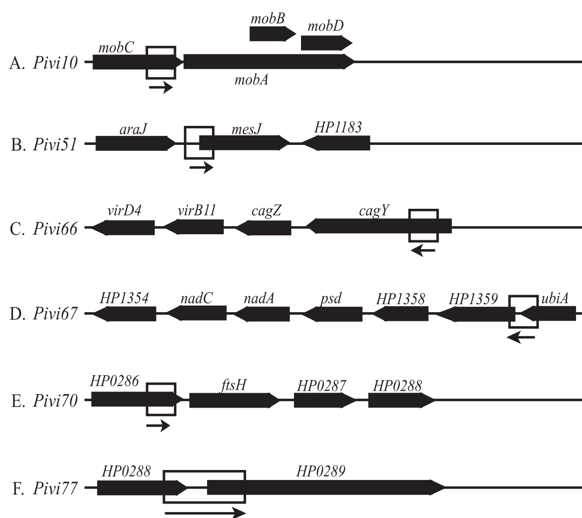

FIG. 4. Locations of in vivo-induced promoters on the *H. pylori* chromosome or an endogenous plasmid. Each in vivo-induced promoter is represented by an open box, and the arrow below the box indicates the direction in which the promoter was cloned. The open reading frames overlapped by and in the genomic region of the in vivo-induced promoters are indicated by thick arrows. (A) P*ivi10* is 153 bp long and overlaps the *mobC* gene in the *H. pylori* strain mG27 endogenous plasmid. It likely regulates *mobA*, *mobB*, and *mobD*. (B) P*ivi51* is 95 bp long and overlaps *mesJ* and the intergenic region upstream of *mesJ* and thus likely regulates *mesJ*. (C) P*ivi66* is 166 bp long and is located within *cagY*, upstream of a putative operon containing *cagZ*, *virB11*, and *virD4*. (D) P*ivi67* is 133 bp long and overlaps HP1359, *ubiA*, and the intergenic region between them. There are six genes that are potentially regulated by P*ivi67*, including three uncharacterized open reading frames (HP1359, HP1358, and HP1354) *psd*, *nadA*, and *nadC*. (E) P*ivi70* is 119 bp long and is located within HP0286. P*ivi70* potentially regulates the metalloprotease gene *ftsH* and uncharacterized open reading frames HP0287 and HP0288. (F) P*ivi77* is 1,679 bp long and overlaps HP0288, HP0289, and the intergenic region between them. P*ivi77* likely regulates the *vacA* paralogue encoded by HP0289.

strains, as was done in the screening of our *H. pylori* RIVET library. Alternatively, retesting of only one Km^s colony for each unique P*ivi* clone could have contributed to a high false-negative rate. We did not perform additional studies to distinguish between these possibilities.

Genomic location of in vivo-induced promoters. We modified the RIVET system for use with *H. pylori* so that it identified in vivo-induced promoters instead of specific in vivo-induced genes. Thus, we were interested in determining the genes regulated by our in vivo-induced promoters. To identify these genes, we mapped the genomic locations of the six P*ivi* clones on the *H. pylori* genome by comparing their sequences to the sequence of the *H. pylori* strain G27 genome (Fig. 4 and unpublished data). The locations of the P*ivi* clones in the G27 genome were the same as those in the previously described *H. pylori* genomes with the exception of the location of P*ivi10*, which is located on an endogenous plasmid (1, 61, 85). Therefore, we used *H. pylori* strain 26695 annotation for all P*ivi* clones except P*ivi10* (85). The gene(s) regulated by each P*ivi* clone is discussed below.

P*ivi* clones regulating *H. pylori* genes involved in interactions with other cells. Three of the P*ivi* clones were located in DNA regions upstream of a gene or set of genes potentially used by *H. pylori* to interact with other bacterial cells or with host cells. P*ivi10* was located in the putative *mobC* gene upstream of the putative *mobA*, *mobB*, and *mobD* genes on an endogenous *H. pylori* strain G27 plasmid (Fig. 4). About 50% of *H. pylori* strains harbor endogenous plasmids (69), and plasmid DNA would have been isolated by our genomic DNA preparation procedure and thus included in our p*cat-T-lib-tnpR* and *H. pylori* RIVET libraries. *mob* genes encode relaxosome components required for plasmid nicking and mobilization during horizontal gene transfer (32). P*ivi66* mapped to the CAG pathogenicity island in the *cagY* gene upstream of a predicted operon containing *cagZ*, *virB11*, and *virD4*. The CAG pathogenicity island is a 40-kb region containing ~30 genes, and its presence is associated with *H. pylori* infections that have more severe disease outcomes (21). Some of the gene products encoded by the CAG pathogenicity island form a type IV secretion apparatus known to inject at least two effectors, CagA and peptidoglycan, into host cells (5, 88). While VirD4 and VirB11 are homologues of an adapter protein likely involved in substrate export (2, 85) and an ATPase that generates energy for apparatus assembly and substrate export (77, 85), respectively, the function of CagZ is not known yet. Finally, P*ivi77* overlapped the intergenic region upstream of one of the uncharacterized *vacA* paralogues, HP0289 (85). *vacA* encodes a well-characterized cytotoxic vacuolating protein belonging to the autotransporter family and is required by *H. pylori* for full virulence (72, 90). The similarity of the HP0289 protein to VacA is concentrated mostly in the carboxy-terminal autotransporter domain rather than in the cytotoxic vacuolating amino-terminal domain (55), and it is therefore not clear what role the HP0289 protein may play in *H. pylori* virulence.

P*ivi51* regulates the putative *mesJ* lysidine synthetase. P*ivi51* overlaps *mesJ* and the upstream intergenic region and therefore likely regulates *mesJ*. *mesJ*, also called *tilS* in *E. coli*, is a member of the PP-loop ATPase superfamily. Members of this superfamily of proteins have distinct enzymatic functions but share an ATP pyrophosphatase domain that targets the alpha-beta bond of ATP (13). Recently, *tilS* was found to govern both the codon and amino acid specificities of the isoleucine tRNA (81). TilS is a lysidine synthetase that generates the lysidine modification at the wobble position of the $tRNA^{Ile}$ anticodon. This changes the codon specificity from AUG to AUA and the amino acid specificity from methionine to isoleucine (81).

P*ivi70* regulates a putative metalloprotease. Another of the in vivo-induced promoters, P*ivi70*, was located in open reading frame HP0285, upstream of the *H. pylori ftsH* gene and two hypothetical open reading frames, HP0287 and HP0288. *ftsH* (HP0286) encodes a putative ATP-dependent metalloprotease (33) and is essential for growth in *E. coli*. In *E. coli* this integral inner membrane metalloprotease degrades both cytosolic proteins, including sigma 32 and lambda CII (36, 37), and transmembrane proteins, including SecY and YccA (43, 44). FtsH likely plays a role in cell division as an *E. coli* strain with a temperature-sensitive *ftsH* allele exhibits filamentous growth at restrictive temperatures (74). Two *ftsH* homologues, HP0286 and HP1069, are present in *H. pylori*; Ge and Taylor (33) showed that HP1069 is essential. There have been no studies to date of HP0286-encoded FstH, and *H. pylori* FtsH targets have not been identified yet. The products of HP0287 and HP0288,

TABLE 3. In vitro growth competition between the *mobABD* and *cagZ* mutants and the correspoding isogenic wild-type strains

Strains	Ratio of mutant to wild type at[a]:						
	Zero time	3 h	6 h	12 h	18 h	24 h	30 h
SS1 Δ*cagZ* + wild type	0.7		0.7	1.4	0.4	0.5	0.2
mG27 Δ*mobABD* + wild type	0.1	1.2	0.5	0.7	0.9	0.9	

[a] Ratios of the mutant to the wild type in an in vitro growth competition experiment at different times. Strains were grown BB10 with shaking under microaerobic conditions. The wild-type strains are isogenic with the corresponding mutants. Each competition assay was repeated two times, and similar results were obtained. The results for one replicate for each mutant are shown.

which are two hypothetical open reading frames located downstream of *fstH*, show no homology to previously identified proteins (85).

P*ivi67* regulates previously uncharacterized proteins. P*ivi67*, the last of the in vivo-induced promoters identified in our analysis, was located upstream of six open reading frames predicted to be in the same operon. These open reading frames included two hypothetical open reading frames, HP1359 and HP1358, followed by *psd* (encoding a phosphatidyl serine decarboxylase), *nadA* (encoding a quinolinate synthetase A), *nadC* (encoding a nicotinate dinucleotide pyrophosphorylase), and an open reading frame encoding a putative adenine-specific methyltransferase.

Genes regulated by P*ivi* clones play a role in *H. pylori* colonization of the host. To address our goal of identifying *H. pylori* genes required for host colonization and/or disease development, we created deletion mutants with mutations in genes potentially regulated by our P*ivi* clones and analyzed these mutants to determine mouse colonization phenotypes. When there was more than one gene potentially regulated by our P*ivi* clones, we attempted to construct a mutant with a deletion of the gene immediately downstream of the promoter. Thus, we created mutants with deletions of the *mobA* and *cagZ* genes, which were immediately downstream of P*ivi10* and P*ivi66*, respectively. However, since the *mobB* and *mobD* open reading frames are located in the *mobA* open reading frame, we actually created a *mobABD* triple-deletion strain. Several unsuccessful attempts were made to delete HP1359, the hypothetical open reading frame downstream of P*ivi67*, and *ftsH*, the metalloprotease gene downstream of P*ivi70*. Our inability to delete these genes is consistent with the hypothesis that they are essential for viability, as proposed by other workers (73). Finally, we did not analyze HP0289 and *mesJ*, the open reading frames downstream of P*ivi77* and P*ivi51*, respectively.

H. pylori nonpolar deletion mutants were constructed by replacing the open reading frame of interest with the *cat* gene, which conferred Cm[r] (see Materials and Methods). Deletion and insertion cassettes were generated using a sewing PCR strategy described by Chalker et al. (22) that allows efficient gene deletion and replacement with a *cat* gene. The *cagZ* gene was deleted in the *H. pylori* SS1 strain commonly used for analyzing *H. pylori* phenotypes in mice because this strain reproducibly infects mice. To address the possibility that *cagZ* is differently regulated in the SS1 strain, we verified that the promoter regulating *cagZ* was similarly induced in the mG27 and SS1 strains, i.e., 77.3 and 76%, respectively (Fig. 3). The *mobABD* genes were deleted in mG27 since we knew that this strain contains the endogenous plasmid from which P*ivi10* was isolated. mG27 infects mice at levels that are approximately 10-fold less than the *H. pylori* SS1 strain levels. In each case, Cm[r] transformants were selected and analyzed by PCR to verify deletion of the *cagZ* and *mobABD* loci. Deletion of *cagZ* and *mobABD* were unlikely to have polar effects on downstream genes, as we verified expression of the gene downstream of *cagZ* in the Δ*cagZ* strain by reverse transcription-PCR (data not shown) and there is a putative transcriptional terminator downstream of *mobABD* (38). Finally, deletion of *mobABD* and deletion of *cagZ* did not confer in vitro growth defects when we used the mutant strains in in vitro competition assays with the wild-type mG27 and SS1 strains, respectively (Table 3).

To assess the contribution of *cagZ* and *mobABD* to *H. pylori* colonization of mice, we carried out both single-strain infection studies and coinfection studies. For single-strain infection studies we infected groups of five mice with each mutant strain (Δ*cagZ* or Δ*mobABD*) and each wild-type strain (SS1 or mG27). For the coinfection studies a mutant strain (Δ*cagZ* or Δ*mobABD*) was used to infect a group of five mice along with the wild-type passage control strain (SS1 and mG27, respectively). Single-strain studies and coinfection studies for each mutant–wild-type strain pair were carried out simultaneously using the same mutant strain and wild-type strain inocula so we could be confident of the mutant strain behavior in the coinfection study.

When it was used as the only infecting strain, the Δ*cagZ* strain had a colonization fitness similar to that of the wild-type strain (Fig. 5). Interestingly, however, when mice were infected with the Δ*cagZ* strain along with the wild-type strain, the

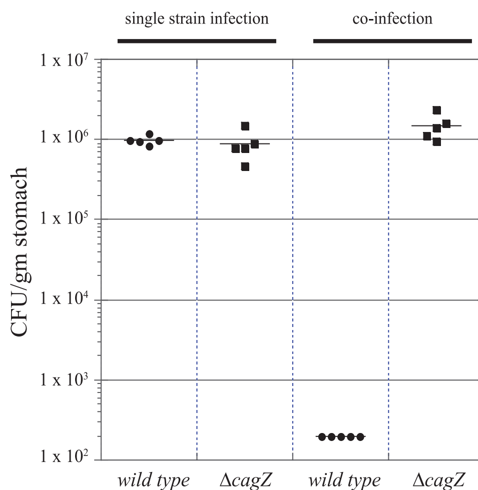

FIG. 5. The Δ*cagZ H. pylori* mutant outcompetes the wild-type strain in a coinfection colonization assay. Single-strain infection and coinfection studies with *H. pylori* Δ*cagZ* and wild-type strains were carried out using FVB/N mice for 2 weeks as described in Materials and Methods. The results of one representative set of experiments are shown. Each point represents one mouse stomach, and the solid lines indicate the averages. When the Δ*cagZ* mutant strain was coinfected with the wild-type strain, we were unable to detect any wild-type strain and thus placed its concentration at the limit of detection (200 CFU/g). This suggests that the Δ*cagZ* mutant strain outcompeted the wild-type strain for colonization (competitive index, 5,713).

FIG. 6. The Δ*mobABD H. pylori* mutant is defective for host colonization. Single-strain infection and coinfection studies with *H. pylori* Δ*mobABD* and wild-type strains were carried out using FVB/N mice for 2 weeks. Each point represents one mouse stomach, and the solid lines indicate the averages. The Δ*mobABD* mutant was not recovered from stomach plates prepared for mice coinfected with both the Δ*mobABD* mutant and wild-type strain, indicating that it was outcompeted by the wild-type strain (competitive index, 0.007).

Δ*cagZ* strain outcompeted the wild-type strain; no wild-type cells or a reduced number of wild-type cells were isolated from the stomachs of mice coinfected with the wild-type and Δ*cagZ* strains (Fig. 5 and data not shown). We calculated that the competitive indices were 5,713 for one coinfection experiment (Fig. 5) and 20 for a second coinfection experiment. These competitive indices suggest that the Δ*cagZ* strain outcompetes the wild-type strain in mice by approximately 1 to 3 orders of magnitude. These results intriguingly suggest that elevated transcription of *cagZ* in mice actually decreases the bacterium's colonization ability, and they are consistent with the notion that genes identified by our RIVET analysis play roles in host colonization.

When the Δ*mobABD* strain was used as the only infecting strain in mice, overall it infected the animals less well than the wild-type strain (Fig. 6). Only three of the five mice treated with the Δ*mobABD* strain became infected, and the mice that were infected had a bacterial stomach load (CFU/g) that was about 0.5 log lower than that of mice infected with the wild-type strain. Consistent with this defect, the Δ*mobABD* mutant strain was not detected in plates containing stomachs of mice coinfected with the Δ*mobABD* mutant and wild-type *H. pylori* strains (competitive index, 0.007). Since the Δ*mobABD* strain performed like the wild-type strain in in vitro growth and competition studies (Table 3), these results suggest that the MobABD proteins are important for promoting stomach colonization. However, complementation studies in which we reintroduced the *mobABD* and *cagZ* genes into our Δ*mobABD* and Δ*cagZ* mutant strains, respectively, are required to be certain that these genes are responsible for the stomach colonization phenotypes, although these mutant strains had no other detectable defects (see Materials and Methods).

DISCUSSION

In this study, we used RIVET with *H. pylori* and identified six promoters that are induced in response to murine host stomachs. We created deletions in two of the genes putatively regulated by these in vivo-induced promoters and found that they are important for host colonization. The Δ*mobABD* mutant strain was defective for host colonization in both single-infection and coinfection studies, and interestingly, the Δ*cagZ* mutant strain outcompeted the wild-type control strain in coinfection studies. Our findings support the hypothesis that *H. pylori* factors important for colonization can be successfully identified as in vivo-induced genes using the RIVET system.

RIVET analysis of *H. pylori*. RIVET has proven to be a valuable tool for identifying bacterial genes important for growth in specific niches, such as inside a host (14, 18, 51, 76, 87). The use of RIVET in this study was the first time that a screen of this kind has been done with *H. pylori*. The advantage of RIVET over other in vivo expression technology systems is that it detects transient gene induction in a small number of cells. RIVET also allows identification of niche-regulated genes expressed at different levels (18, 63). In our RIVET analysis, we combined the *tnpR* allele with a wild-type ribosome-binding site and the *res1* allele, which was recombined with 10-fold-reduced efficiency compared to the wild-type *res* allele (48, 59). This combination was used to identify *H. pylori* genes that were expressed in the lab, induced in the mouse stomach environment, and are important for mouse colonization.

RIVET identifies novel and previously identified host-induced genes. Several previous studies identified *H. pylori* host-induced genes with the goal of finding candidate colonization and disease-promoting factors (11, 12, 34, 45, 78). These studies were carried out both in vivo using humans, Rhesus macaques, and Mongolian gerbils and in vitro using a gastric epithelial cell line (11, 12, 34, 45, 78). Only our P*ivi66* promoter was also identified by these studies. P*ivi66* maps to the CAG pathogenicity island and regulates a predicted operon including HP0524 (*virD4*), HP0525 (*virB11*), and HP0526 (*cagZ*). HP0524 is induced in the Rhesus macaque infection model, and HP0525 is induced in both the Rhesus macaque and Mongolian gerbil infection models (11, 78). This small overlap in the identification of host-induced genes between RIVET and previous studies both validates the RIVET method and highlights the conclusion that RIVET identified a set of unique host-induced genes.

P*ivi66* regulates CAG pathogenicity island genes. P*ivi66* putatively regulates a set of three genes, HP0524 (*virD4*), HP0525 (*virB11*), and HP0526 (*cagZ*), that are located in the *H. pylori* CAG pathogenicity island. Most of the genes in the CAG pathogenicity island, including *virD4*, *virB11*, and *cagZ*, have been analyzed to examine their roles in host colonization, interleukin-8 (IL-8) induction, and CagA transport (30). Interestingly, although *virD4*, *virB11*, and *cagZ* are predicted to be in an operon, their contribution to *cag* pilus function and mouse colonization appears to be distinct. VirD4 is not required for IL-8 induction, but it is important for CagA transport into gastric epithelial cells (30). VirB11 is important for both IL-8 induction and CagA transport into gastric epithelial cells. CagZ contributes to both *cag* functions as the Δ*cagZ* strain induces IL-8 and transports CagA at reduced levels

compared to wild-type *H. pylori* strains (30). Both the Δ*virD4* and Δ*virB11* mutant *H. pylori* strains were attenuated for mouse colonization in single-infection studies, while the Δ*cagZ* strain infected mice at levels similar to the levels of infection of the wild-type strain in single-strain infection studies (56; this study). We observed, however, that the Δ*cagZ* strain had a colonization advantage during coinfection. It is not clear that the reduced *cag* function of the Δ*cagZ* mutant is responsible for the enhanced colonization phenotype that we observed.

The crystal structure and protein interaction profile of CagZ are consistent with a chaperonelike function for CagZ (20), because of the presence of a disordered carboxy-terminal tail and a highly negatively charged surface. Members of a class of chaperones important for delivery of type III effectors, including CesT from enterohemorrhagic *E. coli* and SigE from *Salmonella* spp., have a unique structure, but they also have negatively charged surfaces (52). It is possible that CagZ contributes directly to the transport of the CagA effector; however, since CagZ is not absolutely required for CagA transport, this seems unlikely. Another possibility is that CagZ has a chaperonelike function for assembly of the *cag* secretion pilus. In fact, CagZ was found to interact with 10 Cag proteins (CagY, CagX, CagV, CagT, CagS, CagM, CagI, CagG, CagF, and CagE) in yeast two-hybrid and coimmunoprecipitation studies (16). Many of these proteins form the channel or core of the *cag* pilus structure (4). We hypothesize that CagZ may in fact provide chaperone activity and help assemble antigens, similar to the role proposed for CagY (70). If this prediction is correct, *cagZ* mutants would have less CAG pilus antigenicity and might have been able to avoid an anti-CAG immune response that would have targeted the wild-type strain. Such immune avoidance could have conferred the enhanced colonization phenotype. Future Cag protein assembly experiments with a Δ*cagZ* strain will address this hypothesis.

In vivo-induced promoter regulates genes with putative roles in horizontal gene transfer. DNA transfer via conjugation is a common mechanism for sharing genetic material that can contribute to the success of pathogenic bacteria (26). Conjugation is the transfer of DNA from a bacterial donor to a recipient cell by direct cell-to-cell contact. We identified a group of *H. pylori* endogenous plasmid-borne genes, *mobA*, *mobB*, and *mobD*, as genes that are induced during mouse infection and are important for mouse colonization. The *mob* genes have been shown to be important for DNA transfer via conjugation in other microbes. The *mob* genes encode a relaxase (*mobA*) and accessory proteins (*mob* and *mobD*) that make up a complex called the relaxosome. The relaxosome functions by (i) nicking the DNA molecule to be transferred at the origin of transfer, (ii) becoming covalently associated with the 5′ end of the single-stranded DNA, (iii) transporting the DNA molecule to the conjugation machinery at the inner cell membrane via a coupling protein, and (iv) transporting the DNA molecule across the bacterial membranes through the conjugation machinery into the recipient cell (49). The other components important for DNA transfer via conjugation include the coupling protein mentioned above, the transmembrane protein complex, and the conjugation pilus (7, 50). DNA transfer via conjugation is a widespread method of horizontal gene transfer in the prokaryotic world and has been documented to occur between prokaryotes and eukaryotes as well, including yeast, plant, and mammalian cells (15, 46, 89, 92).

DNA transfer via conjugation has been shown to occur between *H. pylori* clinical isolates and between *H. pylori* and *Campylobacter jejuni* (3, 65). Specific conjugation component homologues have been identified in *H. pylori*, including two chromosomally encoded relaxase proteins, Rlx1 and Rlx2, and two chromosomally encoded coupling proteins, TraG and VirD4 (3). Only Rlx1 and TraG, however, are important for DNA transfer via conjugation (3). Also present in *H. pylori* are three systems ancestrally related to conjugation machinery, including the *cag*, *comB*, and *tfs3* type IV secretions systems (24, 39, 42). Interestingly, none of these three systems appears to be important for conjugation (3). The role of conjugation in *H. pylori* infection biology is not clear yet, however. About 30% of the *H. pylori* clinical isolates characterized contain the *mob* region (38), and it is possible that the *mobA*-encoded relaxase is important for initiating plasmid transfer in some *H. pylori* strains.

In vivo-induced promoter regulates a *vacA* paralogue. Three *vacA* paralogues are present in each of the three published *H. pylori* genomes (the *H. pylori* 26695, J99, and HPAG1 genomes) and in our unpublished genome for the G27 strain (1, 61, 85). The amino termini of the proteins encoded by the *vacA* paralogues (the HP0289, HP0610, and HP0922 proteins) are not well conserved among the paralogues, and each paralogue is not well conserved among the sequenced *H. pylori* strains (1, 61, 85). Like VacA, these proteins belong to the autotransporter protein family based on primary sequence homology. Autotransporters are characterized by three domains, (i) a *sec* signal peptide for transport across the cytoplasmic membrane, (ii) an amino terminus that confers a unique catalytic function, and (iii) a β domain that forms a porelike structure in the outer membrane and transports the amino terminus across the outer membrane. The amino-terminal domain then either remains associated with the outer membrane or is cleaved and secreted, as observed for VacA (29). Most of the homology between the *vacA* paralogues and *vacA* is in the sequences encoding the carboxy-terminal β domains. The HP0289, HP0610, and HP0922 proteins are therefore unlikely to have the same cytotoxic activity as VacA. Based on their primary sequences, these proteins also lack the cleavage site that would suggest that they are secreted into the extracellular milieu (85).

Although the function of the *vacA* paralogues in *H. pylori* virulence is not evident from their primary sequences, recent studies have suggested that they are important for colonization. The identification of HP0289 in a signature-tagged mutagenesis screen for gerbil colonization mutants suggests that HP0289 has a role in colonization (41). Another of the *vacA* paralogues, HP0610, was also found to be important for colonization in a murine model (6). The fact that the third *vacA* paralogue, HP0922, was not identified by either of these screens may indicate that it is not important for animal colonization, but it more likely reflects the difficulty of generating completely saturating screens for *H. pylori*. Additionally, future analyses of the sequences encoding amino-terminal passenger domains of the *vacA* paralogues may reveal their contributions to host colonization.

In vivo-induced promoters regulate essential genes. Three of the in vivo-induced promoters identified in this analysis

regulate *H. pylori* genes that are likely essential for in vitro growth. Our unsuccessful attempts to create deletions in HP1359 and *ftsH* found downstream of P*ivi67* and P*ivi70*, respectively, suggest that these genes are essential, as proposed by other workers (73). Although analysis of essential genes to determine their contributions to *H. pylori* colonization and virulence poses a unique set of challenges, these genes may produce a novel set of *H. pylori* virulence factors. The recent development of an inducible gene expression system for *H. pylori* should expedite the study of these essential genes (10).

In summary, development and use of RIVET for *H. pylori* identified six sets of genes, two of which are important in animal colonization, as shown here. Further, the development of RIVET for *H. pylori* should provide powerful tools for studying *H. pylori* gene expression and gene regulation in the host environment.

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