Recombination-based In Vivo Expression Technology Identifies *Helicobacter pylori* Genes Important for Host Colonization

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**Running Title**

RIVET identifies *H. pylori* colonization factors
Abstract

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 relative to under laboratory conditions. Three of these promoters, named Pivi10, Pivi66, and Pivi77, regulate genes *H. pylori* may use to interact with other microbes or the host. Pivi10 likely regulates the *mobA*, *mobB*, and *mobD* genes, which have potential roles in horizontal gene transfer through plasmid mobilization. Pivi66 occurs within 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*. Pivi77 likely regulates *HP0289*, an uncharacterized paralogue of the *vacA* cytotoxin. We assessed the colonization role of a subset of these genes by creating deletion mutants and analyzing them in single strain and co-infection experiments. We found that the *mobABD* mutant is defective for murine host colonization and the *cagZ* mutant out-competed the wild-type strain in a co-infection analysis. Our work supports that RIVET is a valuable tool for identifying *H. pylori* factors with roles in host colonization.

Introduction

*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 mucosal associated lymphoid tissue (MALT) lymphoma (67, 86). Variability in genetics of both infecting *H. pylori* strains and the
infected hosts likely contribute to the wide range of disease outcomes (8). The goal of this work is to identify additional *H. pylori* virulence factors that contribute to host colonization and/or to disease development and characterize their role in virulence.

Several virulence factors that aid *H. pylori* in colonization of the host and contribute to disease development have been identified through a variety of methods. One of these is the urease enzyme that *H. pylori* requires to survive the low pH of the gastric lumen as it makes its way to the more neutral pH of the gastric mucosa (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 flo (40, 54). Although not required for host colonization, the *H. pylori* virulence factor NapA (neutrophil activating protein) 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 (outer inflammatory protein A) (91). Finally, a quite well known *H. pylori* virulence factor is the Cytotoxin Associated Gene (CAG) pathogenicity island and the effector CagA. Presence of the type IV secretion apparatus encoded by CAG 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 semi-global screens have been carried out to identify additional \textit{H. pylori} genes required for host colonization and/or that contribute to gastric disease. Two studies used \textit{in vivo} screens with libraries of transposon-mutagenized \textit{H. pylori} strains to identify genes required for \textit{H. pylori} host colonization (6, 41). In both of these cases, the \textit{H. pylori} strains used to infect the host were compared with those recovered from the stomach to find genes required for viability in the host (6, 41). These studies identified some genes in common and some unique genes. Two additional studies have been done focusing on \textit{H. pylori} genes induced in response to the host. Two of the known \textit{H. pylori} virulence factors, UreA and NapA, are induced during infection of the host (9), supporting that this strategy is a good one for identifying other virulence factors. One study used \textbf{Selective Capture Of Transcribed Sequences (SCOTS)} analysis to isolate \textit{H. pylori} transcripts induced in human biopsies and experimentally infected gerbils over their expression in culture (34). A second used microarrays to survey \textit{in vivo} \textit{H. pylori} gene expression changes compared to \textit{in vitro} culture conditions (78). Although these analyses have identified genes induced during host infection, they each have their limitations. For example, the transposon mutagenesis screens do not analyze essential genes and the transcript induction screens only identify genes that are induced at the time of RNA isolation. Thus our goal was to identify new \textit{H. pylori} virulence factors that may have been missed by these analyses.

Here we describe the use of \textbf{Recombination-based In Vivo Expression Technology (RIVET)} to identify \textit{H. pylori} promoters induced in response to the murine host. RIVET
technology 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 in *Vibrio cholerae, Lactobacillus plantarum, Staphylococcus aureus,
Mycobacterium tuberculosis*, and *Bordetella pertussis* (14, 18, 51, 76, 87). Herein we
describe how we modified this system for operation in *H. pylori*. Using the RIVET
system, we screened a ~3,000-member library of potential *H. pylori* promoters in mice
and found six to be reliably host-induced. Genes regulated by these promoters include
three with potential roles in *H. pylori* secretion systems; they 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
gene deletion mutants of *mobABD* and *cagZ*. We found that the *H. pylori ΔmobABD*
mutant is defective for host colonization while the *ΔcagZ* mutant actually out-competes
the wild-type parent strain in a competition co-infection analyses. Our work supports 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.** The *H. pylori* strain
mG27 is a mouse-adapted descendent of the clinical isolate G27 (19, 23). mG27 was
generated by serially passaging the G27 *H. pylori* strain through 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 grown at 37°C under microaerobic conditions with a gas mixture of 5-10% O₂, 10%CO₂, and 80-85% N₂. Selective antibiotics for *H. pylori* were added at either 13µg/ml for chloramphenicol or 15µg/ml for kanamycin. *E. coli* was cultured on Luria Bertani (LB) agar plates or liquid media containing ampicillin (Amp) added to a final concentration of 100µg/ml. *E. coli* strains were stored at -80°C in 25-40% glycerol. *H. pylori* strains were stored at -80°C in brain heart infusion media (BHI) supplemented with 10% fetal bovine serum (FBS), 1% (wt/vol) β-cyclodextrin, 25% glycerol, and 5% dimethyl sulfoxide.

**Plasmid construction: pcat-T-tnpR.** To identify promoters that are induced during animal infection, we created a plasmid with a promoterless *tnpR* gene that will recombine onto the *H. pylori* chromosome (Fig. 2A). This plasmid construct contains the *Campylobacter coli* gene for chloramphenicol resistance (*cat*), a strong *E. coli* transcriptional terminator (*rrnBT*T₂)(62) and a promoterless *tnpR*, all flanked by sequences of the *H. pylori* rdxA gene. *cat* is transcribed from its own promoter and the strong *E. coli* terminator *rrnBT*T₂, prevents read-through transcription into *tnpR* (19). This construct directs recombination into the middle of the *rdxA* locus on the *H. pylori* chromosome (80). *rdxA* was used because its loss does not alter *in vitro* growth rates, or 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.

**Plasmid construction: pAW2rkr2.** The *res1-Km-res1* cassette was generated by cloning the *Campylobacter coli aphA3* gene that confers Km*”R”* 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 is called pAW2rkr2 (19). Plasmid pAW2rkr2 targets res1-Km-res1 to the HP0294/HP0295 intergenic locus. Strain mG27 bearing this integration is called ACHP17 (Table 1). Studies of *H. pylori* strains mG27 or SS1 bearing this insertion indicate the modification has no deleterious effects on either growth or mouse colonization (data not shown).

**Generating the library of potential promoters, pcat-T-lib-tnpR.** We generated a library of potential promoters by ligating partially-digested Sau3A genomic DNA isolated from *H. pylori* strain mG27 into the BglII site of the pcat-T-tnpR vector (Fig. 2A). We selected Sau3A DNA fragments from 1-4 kbp for the library by agarose gel purification. These ligated plasmids were electroporated into *E. coli* (DH10B) and plated to LB-Amp+ media. ~12,000 individual AmpR colonies were pooled, amplified by growth in LB-Amp+ broth and DNA was isolated using a midiprep kit (Qiagen) to generate pcat-T-lib-tnpR.

**Generating the *H. pylori* RIVET library.** To create the *H. pylori* RIVET library, pcat-T-lib-tnpR was used to transform *H. pylori* strain ACHP17 (mG27 HP0294/5::res1-Km-res1; Table 1) to chloramphenicol resistance (CmR, Fig. 2B). To minimize the number of *in vitro* expressed promoter-containing clones in our *H. pylori* RIVET library, we passed CmR transformants on CHBA-Cm+ twice before selecting for kanamycin resistance (KmR). This step allowed for *in vitro* expressed clones to transcribe tnpR, resolve res1-Km-res1 and convert to KmS (Fig. 2C). Clones that did not express tnpR in the lab remained KmR and were used to generate frozen stocks for our *H. pylori* RIVET library. Independent library clones were pooled in batches of ten and stored at -80°C. The *H. pylori* RIVET library consists of ~3,000 clones.
Analysis of pcat-\textit{T-lib-tnpR} and the \textit{H. pylori} RIVET library. Both pcat-\textit{T-lib-tnpR} and the \textit{H. pylori} RIVET library were analyzed for diversity and percentage of \textit{H. pylori} genome coverage. We analyzed thirty clones from pcat-\textit{T-lib-tnpR} and thirty clones from the \textit{H. pylori} RIVET library by isolating either plasmid DNA or genomic DNA, respectfully. The cloned \textit{H. pylori} DNA was amplified from these templates by PCR using oligos rrnB1 or catseqst combined with tnpRbk75 (Table 2). The presence and approximate size of the cloned \textit{H. pylori} DNA fragments were estimated by agarose gel electrophoresis. Cloned fragments of similar size were sequenced to determine their uniqueness (Berkeley DNA Sequencing Facility, Berkeley CA). \textit{H. pylori} genome coverage conferred by pcat-\textit{T-lib-tnpR} and the \textit{H. pylori} RIVET library were determined using $N = \ln (1 - P / \ln (1 - I / G))$ where $P$ = probability of obtaining all clones (we used 99%), $I$ = insert size (we used our calculated average insert size, 1.0 kB for pcat-\textit{T-lib-tnpR} and 0.65 kB for the \textit{H. pylori} RIVET library) and $G$ = genome size (1.8 x $10^3$ kB) (Current Protocols in Molecular Biology). Based on these equations, pcat-\textit{T-lib-tnpR} covered the \textit{H. pylori} genome ~4.7 times and the \textit{H. pylori} RIVET library covered the \textit{H. pylori} genome by 84%.

Screening \textit{H. pylori} RIVET library for \textit{in vivo} induced promoters. All animal protocols were approved by the Institutional Animal Use and Care Committee. Fifty \textit{H. pylori} RIVET strains were screened simultaneously in each of two FVB/N mice (Charles River) for promoter induction (Fig. 2D). Five groups of ten \textit{H. pylori} RIVET strains were grown on CHBA+Km to maintain Km$^R$. Immediately prior to mouse infection, the fifty strains were resuspended in BB10 liquid. Bacterial concentration was determined by measuring optical density at 600 nm (OD$_{600}$) and culture volume was adjusted with
BB10 to achieve a bacterial concentration of ~5 x 10\(^7\) cells/ml. Approximately 1 ml of the RIVET strain mixture was used to infect each of two FVB/N mice age 4-6 weeks old by oral gavage (20 gauge, 38 mm length; Popper). Infections were allowed to persist for two weeks after which time we harvested and processed the mouse stomachs as described in Ottemann and Lowenthal (64). In brief, the stomachs were homogenized in 500 µl BB10 using a sterile pestle and dilutions were plated to CHBA plates. Plates were incubated for four days at 37°C under microaerobic conditions. \textit{H. pylori} colonies from these plates were then replica-plated to both CHBA and CHBA+Km plates to identify Km\(^S\) \textit{H. pylori} RIVET strains that induced expression of \textit{tnpR in vivo}. Km\(^S\) RIVET strains were isolated from the corresponding CHBA plate for further analysis (Fig. 2D).

These strains were designated \textit{ivi} plus a number for \textit{in vivo} induced RIVET strain and the \textit{H. pylori} DNA cloned upstream of \textit{tnpR} in these strains is designated Pivi for \textit{in vivo} induced promoter.

**Identifying \textit{in vivo} induced promoters.** To obtain the sequence representing each Pivi clone we isolated genomic DNA (Wizard Genomic DNA Purification Kit, Promega) from the Km\(^S\) \textit{H. pylori} RIVET strains isolated from mice. We then amplified the cloned Pivi region using oligos that annealed to the \textit{cat} gene (catseqst) upstream of the cloned region and the \textit{tnpR} gene (tnpRbk75) downstream of the cloned region by PCR (Table 2). The size of the cloned fragments was estimated by agarose gel electrophoresis and exactly determined by sequencing. The Pivi clones were sequenced using the catseqst and tnpRbk75 oligos (University of California Berkeley DNA sequencing facility). Genomic and endogenous plasmid location of the Pivi clones was determined by comparing their sequence to that of the \textit{H. pylori} G27 strain sequenced...
genome (unpublished sequence). Operon analysis was done using the site
http://www.microbesonline.org/.

**Reconstruction of in vivo induced strains.** Since the *H. pylori* RIVET strains
that were induced in the host resolved the res1-Km-res1 cassette (and were therefore
KmS) we reintroduced the res1-Km-res1 cassette into the original locus of each of these
strains prior to our secondary analysis. One KmS mouse-output strain for each of the
thirteen unique KmS ivi strains was naturally transformed with pAW2rkr2.
Transformants were selected based on KmR and proper integration was verified by PCR
using oligos that flank the site of integration, HP0294end and HP0295end (Table 2).

**Testing promoter induction in vitro and in vivo.** Each of the thirteen
reconstructed ivi (ivi#R strains (Table 1)) was analyzed for promoter induction conferred
by the cloned Pivi in the lab and in FVB/N mice. The ivi#R strains were grown on
CHBA+Km prior to these analyses to maintain KmR. *In vitro* promoter induction was
carried out by passing each ivi#R strain on CHBA without Km selection for two weeks;
five passes to fresh CHBA. The *H. pylori* ivi#R cells were then resuspended in BB10
media and plated to CHBA for single colonies (~200 colonies/plate). After incubating
these plates for four days at 37°C under microaerobic conditions, the *H. pylori* colonies
were replica-plated to both CHBA and CHBA+Km plates. Percent promoter induction
was calculated as the number of KmS colonies divided by the total number of colonies x
100. To determine *in vivo* induction conferred by the Pivi regions, each ivi#R strain was
independently used to infect a set of FVB/N mice. After a two-week infection time, the
mouse stomachs were again harvested and plated to CHBA to isolate *H. pylori*. The
plates were incubated for four days at 37°C under microaerobic conditions and then
replica-plated to CHBA and CHBA+Km to determine the number of *H. pylori* cells that had converted to Km$^S$ while in mice. Promoter induction *in vivo* is calculated as the number of Km$^S$ colonies divided by the total number of colonies analyzed x 100. Statistical difference between promoter induction *in vivo* versus *in vitro* was calculated using the two-tailed Student’s t-test.

**Construction of *H. pylori* gene deletion mutants.** We generated gene deletions of genes regulated by our *in vivo* induced promoters by replacing the gene of interest with a nonpolar allele of the *Campylobacter 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 1) one oligo that anneals to the chromosome and 2) another oligo that anneals to the chromosome and either the start or end of the cat gene. A third PCR product representing the nonpolar cat allele was generated using the oligos, catR2 and catF (Table 2). The PCR products representing the upstream chromosomal region, the downstream chromosomal region and the cat gene were generated independently, agarose gel purified (GFX™ PCR DNA and Gel Band Purification Kit, GE Healthcare), and then combined. The mixture of PCR products was used as a template for the oligos that anneal to the far upstream and downstream PCR product regions. The large PCR products generated from these reactions, upstream region-cat-downstream region, were agarose gel purified and used to naturally transform *H. pylori* strains SS1 (47) or mG27 (19) to Cm$^R$ as previously described (72). All mutant strains were found to be wild type for motility by microscopic inspection and wild type for urease activity using a pH indicator buffer (Difco urea broth, Difco)(data not shown).
**Mouse colonization analyses.** *H. pylori* strains for colonization analyses were passed minimally in the lab on CHBA (two to three times) and then either taken from CHBA after growth for ~18 hours or from a BB10 culture grown for ~18 hours. *H. pylori* strains grown on CHBA were transferred to BB10 media prior to infection and analyzed for motility and bacterial cell concentration (OD$_{600}$). *H. pylori* strains grown in BB10 were analyzed directly for motility and bacterial cell concentration (OD$_{600}$). Approximately 1 ml of $5 \times 10^7 – 5 \times 10^8$ CFU/ml *H. pylori* culture was used to inoculate mice by oral gavage. For single infection studies, either the mutant *H. pylori* strain or the appropriate wild-type control strain was used to infect mice. For the co-infection analysis, the mutant and wild-type strains were grown separately and analyzed for motility and bacterial cell concentration (OD$_{600}$) before mixing the cultures for co-infection. The observed bacterial cell concentration was used to generate a mixed culture containing approximately equal cell numbers of the mutant and wild-type strain. The actual bacterial cell concentration was more accurately determined by culture dilution and plating. Infections were allowed to persist for two weeks after which time the mouse stomachs were isolated and plated on CHBA as described previously. The stomachs of mice infected with the mutant strains were plated to CHBA+Cm and the stomachs of mice infected with the wild-type strain were plated to CHBA. The stomachs of mice co-infected with both the mutant and the wild-type strain were plated to both CHBA+Cm and CHBA. The competitive index was calculated as the colony forming units/gm (CFU/gm) of the mutant strain output divided by the CFU/gm wild-type strain output over the CFU/gm mutant strain input divided by the CFU/gm wild-type strain input.
Results

We have adapted the Recombination-based In Vivo Expression Technology (RIVET) system used with great success by Camilli and coworkers (18) for use in H. pylori and used it to identify promoters induced in response to murine stomachs.

Adapting Vibrio cholerae RIVET for use in H. pylori required: a) creation of a H. pylori antibiotic resistance reporter for tnpR recombinase expression flanked by res1 sites. We used kanamycin resistance (res1-Km-res1) and b) creation of an H. pylori genomic/promoter library fused to the tnpR gene (pcat-T-lib-tnpR). When tnpR expression is directed by the cloned H. pylori DNA, TnpR binds the res1 sequences and catalyzes the removal of the intervening kanamycin resistance cassette (Fig. 1).

We modified the original RIVET from a V. cholerae-specific antibiotic resistance (tetracycline) to one that works in H. pylori. For this purpose we chose a kanamycin resistance gene (aphA3, 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 somewhat expressed in vitro but had elevated expression in the host. The res1-Km-res1 cassette was cloned into a plasmid that directed its integration into the chromosomal region between ORFs HP0294 and HP0295 (19).

We integrated this construct into the chromosome of H. pylori strain mG27 to ensure both its presence in single copy and stable maintenance. H. pylori strain mG27 is a mouse-adapted version of the commonly used H. pylori strain G27 (19, 79). Integration at HP0294-HP0295 occurs by a double crossover gene replacement, and does not affect
the growth or virulence of *H. pylori* strains mG27 or SS1 (data not shown). We verified that *res1-Km-res1* integrated into the proper chromosomal region by selection for kanamycin resistant (Km\(^R\)) transformants and by PCR with oligonucleotide primers that flank the insertion site (data not shown). This strain is called *ACHP17* (Table 1).

**Generating the library of potential promoters, pcat-T-lib-tnpR.**

To generate pcat-T-lib-tnpR (Table 1), we created a plasmid with promoterless *tnpR* and cloned a partially digested Sau3A *H. pylori* genomic library upstream of *tnpR* (Fig. 2A). This plasmid contains the *cat* gene, which confers chloramphenical resistance (Cm\(^R\)), followed by a strong *E. coli* terminator, the site for *H. pylori* library insertion and promoterless *tnpR*, all flanked by sequences of *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, with a wild-type ribosome binding site, for our work (48). pcat-T-lib-tnpR is a collection of ~12,000 independent colonies. Our analysis of 30 library clones suggests that 70% contain inserts with an average size of 1.0 kB (data not shown). Our analysis indicates the library is diverse and covers the 1.8 MB *H. pylori* genome 4.7 times (see Materials and Methods).

The *rdxA* sequences flanking *cat-T-lib-tnpR* in pcat-T-lib-tnpR targets its integration to 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, pcat-T-lib-tnpRI was used to transform the *H. pylori* strain *ACHP17* (*HP0294/HP0295:: res1-Km-res1*, Table 1) to
Cm\textsuperscript{R} (Fig. 2B). Each Cm\textsuperscript{R} transformant was passed twice to Cm\textsuperscript{+} media before selection on Km\textsuperscript{+} media and subsequent freezing. We initially passed the Cm\textsuperscript{R} transformants without Km selection to allow promoters that express in the lab to convert the strains carrying them to Km\textsuperscript{S} (Fig. 2C). We were interested in promoters that were not expressed in the lab or expressed at very low levels in the lab and thus in bacteria that retain Km\textsuperscript{R} (\textit{res1-Km-res1}). The genomic DNA of 30 Cm\textsuperscript{R} \textit{H. pylori} transformants was screened by PCR using oligonucleotides that anneal upstream and downstream of the genomic DNA fragment to assess whether this subset of transformants contained unique genomic DNA inserts upstream of \textit{tnpR}. Our screening of the \textit{H. pylori} transformants suggests that 70\% contain unique inserts, and that these inserts were smaller (0.65 kB) than those found for the same library in \textit{E. coli}. Although we do not know for certain, we speculate that the average insert size in the \textit{H. pylori} RIVET library is smaller than that in \textit{pcat-T-lib-tnpR} because either the larger inserts had more potential sites for \textit{H. pylori}'s restriction systems or because larger inserts recombine less efficiently. Our \textit{H. pylori} RIVET library contains 3,340 independent clones that have been pooled in groups of 10 and stored in \textit{H. pylori} freezing media at –80°C. This number of transformants covers ~84\% of the \textit{H. pylori} genome (see Materials and Methods).

**Identifying \textit{H. pylori} host induced promoters**

To identify putative host-induced promoters, we used the \textit{H. pylori} RIVET library strains to infect FVB/N mice (Fig. 2D). We screened 50 \textit{H. pylori} RIVET library strains in parallel by infecting mice with a mixture of 50 RIVET library strains. Salama and co-workers have shown that this pool size allows each strain to independently establish infection of the mouse gastric mucosa (6). The infections were allowed to
persist for two weeks, at which time we sacrificed the animals and harvested their stomachs. The stomachs were homogenized, diluted and plated to Cm\(^+\) media. After four days, each plate was replica-plated to both Cm\(^+\) and Km\(^+\) media (Fig. 2D). RIVET strains that induced \(tnpR\) expression at any point during the infection converted to Km\(^S\). The Km\(^S\) strains were rescued from the corresponding Cm\(^+\) plate and saved as a frozen stock 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 resulted in the identification of 113 Km\(^S\) \(H. pylori\) RIVET strains. By PCR amplification and sequencing of the region cloned upstream of \(tnpR\) in these strains, we determined that the 113 Km\(^S\) strains represent thirteen unique clones. We refer to these unique clones as Pivi for in vivo induced promoter and to the strains containing them as ivi; a number corresponding to the order of ivi strain isolation is also included to refer to a specific clone or strain (Table 1).

**Verifying the P vis are induced in the host.**

We retested the Pivi clones identified above to ensure that their expression in mice is greater than their expression in vitro. The ivi strains, isolated from mice stomachs as Km\(^S\), had induced \(tnpR\) expression in vivo and thus resolved 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, Materials and Methods). Strains containing one of the thirteen P vis fused upstream of \(tnpR\) and the \(res1-Km-res1\) cassette were called ivi\#R and were further analyzed for promoter-directed expression of \(tnpR\) based on their conversion from Km\(^R\) to Km\(^S\) (Table 1).
To determine Pivi-directed expression of tnpR in the host, we infected mice with each reconstructed ivi#R strain as a single infecting strain. The infection was allowed to persist for two weeks, at which time we sacrificed the animals, harvested their stomachs and plated for single colonies on Cm⁺ media as described above. Plates containing the stomach-isolated colonies were then replica-plated to Cm⁺ media and Km⁺ media. In vivo expression of tnpR was assayed as the number of Km⁻ colonies divided by the total number of colonies analyzed x 100 (% promoter induction in vivo). To determine the in vitro expression conferred by each Pivi, each ivi#R strain was passed on Cm⁺ media in the lab for two weeks (4-5 passes) and then plated to Cm⁺ media for single colonies. These plates were replica-plated to both Cm⁺ media and to Km⁺ media to determine the % promoter induction in vitro.

We found that four of the thirteen Pivi clones originally isolated from mice as Km⁻ ivi strains conferred in vivo expression of tnpR that was significantly higher than in vitro expression when tested as the single infecting strain (two-tailed Student’s t-test, Fig. 3). These Pivi clones include Pivi51 (p< 0.001), Pivi66 (p < 0.001), Pivi70 (p< 0.05) and Pivi77 (p < 0.05). Two additional Pivi clones, Pivi10 and Pivi67, were very close to our cut-off for statistical significance with p < 0.1. We have included these six Pivis in our subsequent analyses. The remaining seven Pivi clones identified in our screen as Km⁻ ivi strains did not show in vivo induction of tnpR when retested as single infecting strains (Fig. 3). It is possible that these Pivi clones are either simply not induced in mice and were isolated as background inherent in this screen or are only induced when co-infected with additional strains as was done in the screening of our H. pylori RIVET library. Alternatively, re-testing of only one Km⁻ colony for each unique Pivi could have
contributed to a high false negative rate. We have not performed the additional studies to distinguish between these possibilities.

**Genomic location of in vivo induced promoters.**

Our modification of the RIVET system for use in *H. pylori* was such that it identified *in vivo* induced promoters as opposed to specific *in vivo* induced genes. Thus we were interested to know the genes regulated by our *in vivo* induced promoters. To identify these genes, we mapped the genomic location of the six *Pivis* on the *H. pylori* genome by comparing their sequence to that of the *H. pylori* strain G27 sequenced genome (Fig. 4, unpublished genome). Mapping of the *Pivis* is the same in the published *H. pylori* genomes with the exception of *Pivi10*, which is located on an endogenous plasmid (1, 61, 85). We therefore used *H. pylori* strain 26695 annotation for all *Pivis* except *Pivi10* (85). The gene(s) regulated by each *Pivi* is discussed below.

**Pivis regulating *H. pylori* genes involved in interactions with other cells**

Three of the *Pivis* are 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. *Pivi10* is located within 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 such DNA would have been isolated in our genomic DNA prep and thus included in our pcat-T-lib-tnpR and *H. pylori* RIVET libraries. *mob* genes encode relaxosome components required for plasmid nicking and mobilization during horizontal gene transfer (32). *Pivi66* maps to the cytotoxin-associated gene pathogenicity island (CAG-PAI) within the *cagY* gene upstream of a predicted operon containing *cagZ*, *virB11* and *virD4*. The CAG-PAI is a
40 kB region encoding ~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-PAI 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, CagZ function is yet unknown. Finally, Pivi77 overlaps the intergenic region upstream of one of the uncharacterized *vacA* paralogues, *HP0289* (85). *vacA* encodes a well-characterized cytotoxic vacuolating protein of the autotransporter family and is required by *H. pylori* for full virulence (72, 90). The similarity of HP0289 to VacA is mostly concentrated in the carboxy-terminal autotransporter domain rather than the cytotoxic-vacuolating amino-terminal domain (55) and it is therefore not clear what role HP0289 might play in *H. pylori* virulence.

**Pivi51** regulates the putative *mesJ* lysidine synthetase

*Pivi51* 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 super family. This super family of proteins has 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\(^{\text{Ile}}\) anticodon. This changes the codon specificity from AUG to AUA and the amino acid specificity from methionine to isoleucine (81).

**Pivi70** regulates a putative metalloprotease
Another of the *in vivo* induced promoters Pivi70 maps within open reading frame HP0285, upstream of the *H. pylori* gene *ftsH* 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* 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 (1996) have shown that HP1069 is essential. There have been no studies to date of HP0286 encoded FstH and *H. pylori* FtsH targets have yet to be identified. The two hypothetical open reading frames, HP0287 and HP0288 located downstream of *fstH* show no homology to identified proteins (85).

**Pivi67 regulates previously uncharacterized proteins.**

Pivi67, the last of the *in vivo* induced promoters identified in our analysis is located upstream of six open reading frames predicted to be in the same operon. These include, two hypothetical open reading frames, HP1359 and HP1358, followed by *psd*, a phosphatidyl serine decarboxylase, *nadA*, a quinolinate synthetase A, *nadC*, a nicotinate-dinucleotide pyrophosphorylase, and a putative adenine-specific methyltransferase.

**Genes regulated by Pivis 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 in genes potentially regulated by our Pivis and analyzed these strains for mouse colonization phenotypes. In cases
where there were more than one gene potentially regulated by our Pivis, we attempted to construct a deletion of the gene immediately downstream of the promoter. Thus we have created gene deletions of mobA and cagZ, which are immediately downstream of Pivi10 and Pivi66 respectively. However, since the mobB and mobD open reading frames are contained within the mobA open reading frame, we have actually created the triple mobABD deletion strain. Several unsuccessful attempts were made to delete HP1359, the hypothetical open reading frame downstream of Pivi67 and fisH, the metalloprotease downstream of Pivi70. Our inability to delete these genes is consistent with them being essential for viability as proposed by others (73). Finally, we have yet to analyze HP0289 and mesJ, the open reading frames downstream of Pivi77 and Pivi51, respectively.

H. pylori non-polar deletion mutants were constructed by replacing the open reading frame of interest with the cat gene that confers chloramphenicol resistance (materials and methods). Deletion/insertion cassettes were generated using a sewing PCR strategy as described in Chalker et al. (2001) that allows for efficient gene deletion and replacement with a cat gene. The cagZ gene deletion was carried out in the H. pylori SS1 strain commonly used for analyzing H. pylori phenotypes in mice because it 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, 77.3% and 76% respectively (Fig. 3). The mobABD gene deletion was carried out in mG27 since we know this strain contains the endogenous plasmid from which Pivi10 was isolated. mG27 infects mice to levels approximately 10-fold less than the H. pylori SS1 strain. In each case, CmR transformants were selected and analyzed by
PCR to verify deletion of cagZ and mobABD loci. Deletion of cagZ and mobABD are unlikely to have polar effects on downstream genes as we verified the expression of the gene downstream of cagZ in the ΔcagZ strain by reverse transcription PCR (data not shown) and there is a putative transcriptional terminator downstream of mobABD (38). Finally, deletion of mobABD or cagZ does not confer in vitro growth defects when we analyzed these mutant strains in in vitro competition assays with the wild-type mG27 or SS1 strain, respectively (Table 3).

To assess the contribution of cagZ and mobABD to H. pylori colonization of mice, we carried out both single strain infection and co-infection studies. For single strain infection studies we infected a set of five mice with each mutant strain, ΔcagZ or ΔmobABD, and each wild-type strain, SS1 or mG27. For the co-infection studies each mutant strain, ΔcagZ or ΔmobABD, were co-infected into a set of five mice with the SS1 or mG27 wild-type pass control strain, respectively. Single strain and co-infection studies for each mutant/wild-type strain pair were carried out simultaneously using the same mutant strain and wild-type strain inoculums so we could be confident of the mutant strain behavior in the co-infection study.

When analyzed as the only infecting strain, the ΔcagZ strain had a colonization fitness similar to the wild-type strain (Fig. 5). Interestingly however, when the ΔcagZ strain was co-infected with the wild-type strain, it out-competed the wild-type strain; no or a reduced number of wild-type cells were isolated from mouse stomachs co-infected with the wild-type and ΔcagZ strains (Fig. 5 and data not shown). We calculated a competitive index of 5,713 for one co-infection experiment (Fig. 5) and 20 for a second co-infection experiment. These competitive indices suggest the ΔcagZ strain out-
competes the wild-type strain in mice by approximately one to three orders of magnitude. These results intriguingly suggest that elevated transcription of \textit{cagZ} in mice would actually decrease the bacterium’s colonization ability, and is consistent with the notion that our RIVET analysis-identified genes play roles in host colonization.

When the \textit{ΔmobABD} strain was analyzed as the only infecting strain in mice, it infected overall less well than the wild-type strain (Fig. 6). Only three out of the five mice treated with the \textit{ΔmobABD} strain became infected and those that were infected had a CFU/gm stomach load about one-half log lower than mice infected with the wild-type strain. Consistent with this defect, the \textit{ΔmobABD} mutant strain was not detected in stomach platings of mice co-infected with the \textit{ΔmobABD} mutant and wild-type \textit{H. pylori} strains (CI = 0.007). Since the \textit{ΔmobABD} strain performs similar to the wild-type strain in \textit{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 re-introduce the \textit{mobABD} and \textit{cagZ} genes into our \textit{ΔmobABD} and \textit{ΔcagZ} mutant strains, respectively, will be required to be certain that these genes are responsible for the stomach colonization phenotypes, although these mutant strains had no other detectable defects (see methods).

\textbf{Discussion}

In this study, we used Recombination-based \textit{In Vivo Expression Technology} (RIVET) in \textit{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 \textit{in vivo}-induced promoters and found that they are important for host colonization. The
\( \Delta \text{mobABD} \) mutant strain was defective for host colonization in both single and co-
infection studies and interestingly, the \( \Delta \text{cagZ} \) mutant strain out-competed the wild-type
control strain in co-infection studies. Our findings support that \( H. \text{pylori} \) factors
important for colonization can be successfully identified as \emph{in vivo} induced genes using
the RIVET system.

**RIVET analysis in \( H. \text{pylori} \)**

RIVET has proven to be a valuable tool for identifying bacterial genes important
for growth in specific niches, such as inside of a host (14, 18, 51, 76, 87). Use of RIVET
in this study is the first time a screen of this kind has been done in \( H. \text{pylori} \). The
advantage of RIVET over other IVET systems is that it detects transient gene induction in
a small number of cells. RIVET also allows for the identification of niche-regulated
genes expressed at different levels (18, 63). In our RIVET analysis, we combined the
\( \text{tnpR} \) allele with a wild-type ribosome-binding site and the \( \text{res}1 \) allele that is recombined
with a 10-fold reduced efficiency compared to the wild-type \( \text{res} \) allele (48, 59). This
combination identified \( H. \text{pylori} \) genes that were expressed in the lab and induced in the
mouse stomach environment and important for mouse colonization.

**RIVET identifies novel and previously identified host-induced genes**

Several previous studies identified \( H. \text{pylori} \) 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 \emph{in vivo}, in humans, Rhesus macaque and Mongolian gerbils,
and \emph{in vitro}, in a gastric epithelial cell line (11, 12, 34, 45, 78). Only our \( \text{Pivi}66 \) promoter
was also identified by these studies. \( \text{Pivi}66 \) maps to the CAG-pathogenicity island, and
regulates a predicted operon including \( \text{HP0524} (\text{virD4}), \text{HP0525} (\text{virB11}) \) and \( \text{HP0526} \)
(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 that RIVET has identified a set of unique host-induced genes.

**Pivi66 regulates CAG pathogenicity genes**

Pivi66 putatively regulates a set of three genes, HP0524 (virD4), HP0525 (virB11) and HP0526 (cagZ) located within the *H. pylori* CAG pathogenicity island. Most of the genes in the CAG pathogenicity island, including virD4, virB11, and cagZ, have been analyzed for their roles in host colonization, 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 distinct.

VirD4 is not required for IL-8 induction, but 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 ΔcagZ strain induces IL-8 and transports CagA at reduced levels compared to wild-type *H. pylori* strains (30). Both the ΔvirD4 and ΔvirB11 mutant *H. pylori* strains are attenuated for mouse colonization in single infection studies while the ΔcagZ strain infected mice at levels similar to the wild-type strain in single strain infection studies (56) and this study).

We observed, however, a colonization advantage for the ΔcagZ strain during co-infection. It is not clear that the ΔcagZ mutant reduced cag function is responsible for the enhanced colonization phenotype we observe.
The crystal structure and protein interaction profile of CagZ are consistent with a chaperone-like function for CagZ (20), because of the presence of a disordered carboxy-terminal tail and a highly negatively charged surface. A class of chaperones important for delivery of type III effectors, including CesT from enterohaemorrhagic E. coli and SigE from Salmonella spp., are unique in structure, but also have negatively charged surfaces (52). It is possible that CagZ directly contributes 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 provides chaperone-like function for assembly of the cag secretion pilus. In fact, CagZ was found to interact with ten Cag protein by yeast two-hybrid and co-immunoprecipitation studies, including CagY, CagX, CagV, CagT, CagS, CagM, CagL, CagG, CagF and CagE (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 in a ∆cagZ strain will address this hypothesis.

In vivo induced promoter regulates genes with putative role 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 induced during mouse infection and 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 (*mobB*, *mobD*) that make up a complex called the relaxosome. The relaxosome functions by 1) nicking the DNA molecule to be transferred at the origin of transfer, 2) becoming covalently associated with the 5’ end of the SS DNA, 3) transporting the DNA molecule to the conjugation machinery at the inner cell membrane via a coupling protein and 4) 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 above-mentioned coupling protein, 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, plants 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 type IV secretion systems *cag*, *comB*, and *tfs3* (24, 39, 42). Interestingly, none of these three systems appear important for
conjugation (3). The role of conjugation in *H. pylori* infection biology is not yet clear, 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**

There are three *vacA* paralogues present in each of the three published *H. pylori* genomes (26695, J99, and HPAG1) and in our unpublished genome for the G27 strain (1, 61, 85), unpublished genome). The amino-termini of the proteins encoding the *vacA* paralogues, HP0289, HP0610 and HP0922 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, 1) a *sec*-signal peptide for transport across the cytoplasmic membrane, 2) an amino-terminus that confers a unique catalytic function, and 3) a β-domain that forms a pore like 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 such as for VacA (29). Most of the homology between the *vacA* paralogues and *vacA* lies in their carboxy-terminal β-domains. HP0289, HP0610, and HP0922 are therefore unlikely to have the same cytotoxic activity as VacA. Based on their primary sequence, these proteins also lack the cleavage site that would suggest 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 sequence, recent studies suggest 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 parologue, HP0922 was not identified by either of these screens may indicate that it is not important for animal colonization, but more likely reflects the difficulty with generating completely saturating screens in H. pylori. Additionally, future analyses of the amino-terminal passenger domains of the vacA paralogues may reveal their contribution 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 Pivi67 and Pivi70, respectively, suggests that these genes are essential as proposed by others (73). Although the analysis of essential genes for their contribution to H. pylori colonization and virulence will pose a unique set of challenges, they 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, the development and use of RIVET in H. pylori has identified six sets of genes, two of which we show to be important in animal colonization. Further, the development of RIVET technology in H. pylori will provide us powerful tools to study H. pylori gene expression and gene regulation in the host environment.
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pyrophosphatase domain: implications for the evolution of sequence motifs and


**Table 1. Strains and plasmids used in this study.**

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<td>ΔcagZ</td>
<td>SS1 ΔcagZ::cat</td>
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</table>

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>pcat-T-tnpR</td>
<td>cat gene-E. coli rrnBT1T2-BglII-tnpR</td>
<td>(19)</td>
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<tr>
<td>pAW2rkr2</td>
<td>res1-aphA3-res1 cassette at HindIII site of pMW2</td>
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Table 2. Oligonucleotides used in this study.

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<th>Reference</th>
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<td>catseqst</td>
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<td>This study</td>
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<tr>
<td>catF</td>
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<td>This study</td>
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<tr>
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<tr>
<td>tnpRbk75</td>
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<td>(19)</td>
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<tr>
<td>rrnB1</td>
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<td>(19)</td>
</tr>
<tr>
<td>G27_633_D1</td>
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</tr>
<tr>
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<td>This study</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
<td>HP0295end</td>
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</table>
Table 3. *In vitro* growth competitions between the *mobABD* and *cagZ* mutants and their respective isogenic wild-type strains.

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>0 h.</th>
<th>3 h.</th>
<th>6 h.</th>
<th>12 h.</th>
<th>18 h.</th>
<th>24 h.</th>
<th>30 h.</th>
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</thead>
<tbody>
<tr>
<td>SS1 ΔcagZ + WT</td>
<td>0.7</td>
<td>-</td>
<td>0.7</td>
<td>1.4</td>
<td>0.4</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>mG27 ΔmobABD + WT</td>
<td>0.1</td>
<td>1.2</td>
<td>0.5</td>
<td>0.7</td>
<td>0.9</td>
<td>0.9</td>
<td>-</td>
</tr>
</tbody>
</table>

Ratio of mutant:wild type (WT) in an *in vitro* growth competition at the indicated times. Growth was done in BB10 with shaking under microaerobic conditions. WT strains are isogenic to their mutants. Each competition was repeated two times with similar results; shown is one replicate for each mutant.
Figure legends

Figure 1. Recombination-based In Vivo Expression Technology (RIVET) for use in H. pylori. A) The H. pylori RIVET strain contains two chromosomally located RIVET components, 1) a promoterless tnpR gene that encodes the site-specific recombinase integrated at the H. pylori rdxA locus and 2) a cassette in which the aphA3 gene (also called Km) that confers KmR is cloned between res1 sequences (res1-km-res1) integrated at the HP0294/HP0295 intergenic locus. In the absence of an upstream promoter, tnpR is not expressed and the H. pylori strain remains KmR. B. (1) In the presence of an upstream promoter, P, tnpR is expressed, (2) the TnpR recombinase binds the res1 sequences to catalyze the removal of the Km gene cloned between them, (3) converting the H. pylori strain to KmS.

Figure 2. Construction and screening of the pcat-T-lib-tnpR and H. pylori RIVET libraries. A) The H. pylori RIVET library in E. coli, pcat-T-lib-tnpR, was generated by cloning Sau3A digested H. pylori genomic DNA into the BglII site upstream of tnpR in plasmid pcat-T-tnpR. Approximately 12,000 independent clones were propagated in E. coli with an average insert size of 1.0 Kb. B) The H. pylori RIVET library, pcat-T-lib-tnpR was integrated into the H. pylori rdxA chromosomal locus of H. pylori strain ACHP17 that has the res1-km-res1 cassette at an unlinked chromosomal locus. RIVET-library containing transformants were selected on Cm⁺-media. C) In vitro expressing clones were removed from the H. pylori RIVET library as transformants that converted to KmS in the lab. 3,340 CmR KmR transformants were collected in our H. pylori RIVET library. D) Fifty independent H. pylori RIVET library clones were simultaneously
screened in each of two mice for \textit{in vivo} induction (conversion to \textit{Km}^\text{S}). After two-week
infections, mouse stomachs were harvested and plated to CHBA-Cm\textsuperscript{+} to isolate \textit{H. pylori}.
The CHBA-Cm\textsuperscript{+} plates were then replica-plated to CHBA-Km\textsuperscript{+} media to identify \textit{in vivo}
induced Km\textsuperscript{S} clones.

\textbf{Figure 3. Six RIVET identified promoters were induced in the host.} Relative
promoter induction of each of the thirteen reconstructed RIVET strains isolated from
mice as Km\textsuperscript{S} 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 set of mice
for two weeks (dark bars). Promoter induction in the lab was analyzed after two weeks
of \textit{in vitro} growth on CHBA media (light gray bars). For both \textit{in vivo} and \textit{in vitro}
experiments, promoter induction was calculated as the number of Km\textsuperscript{S} CFU isolated
divided by the total number of CFU, x 100. All promoters marked with asterixes were
induced to a greater extent \textit{in vivo} than \textit{in vitro}. A Two-tailed Student T-test determined
the following p-values for these promoters, comparing \textit{in vitro} to \textit{in vivo} induction:
\textit{Pivi10} (< 0.1), \textit{Pivi51} (<0.001), \textit{Pivi66} (<0.001), \textit{Pivi67} (<1.0), \textit{Pivi70} (<0.05) and
\textit{Pivi77} (<0.05). The other promoters did not differ appreciably between \textit{in vivo} and \textit{in
vitro}. Error bars represent standard deviation. For each RIVET strain, the \textit{in vitro}
experiment was replicated \textit{X} times and the \textit{in vivo} analysis was carried out in \textit{Y} mice,
given as the promoter name followed by (\textit{X,Y}): \textit{Pivi2} (2,5), \textit{Pivi7} (3,5), \textit{Pivi10} (3,4),
\textit{Pivi11} (2,1), \textit{Pivi23} (3,9), \textit{Pivi39} (6,0), \textit{Pivi50} (1,2), \textit{Pivi51} (4,9), \textit{Pivi63} (2,2), \textit{Pivi66}
(6,3), \textit{Pivi67} (2,3), \textit{Pivi70} (2,4), \textit{Pivi77} (4,3).
Figure 4. Location 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 each box indicates the direction 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 dark arrows. A) Pivi10 is 153bp in length and overlaps the mobC gene on the H. pylori mG27 strain endogenous plasmid. It likely regulates mobA, mobB, and mobD. B) Pivi51 is 95bp in length and overlaps mesJ and the intergenic region upstream of mesJ, and thus likely regulates mesJ. C) Pivi66 is 166bp in length and is located within cagY, upstream of a putative operon containing cagZ, virB11 and virD4. D) Pivi67 is 133bp and overlaps HP1359 and ubiA and the intergenic region between them. There are six genes potentially regulated by Pivi67, including three uncharacterized ORFs, HP1359, HP1358, and HP1354 and psd, nadA, and nadC. E) Pivi70 is 119bp in length and is located within HP0286. Pivi70 potentially regulates the metalloprotease fisH and the uncharacterized ORFS, HP0287 and HP0288. F) Pivi77 is 1,679bp in length and overlaps HP0288 and HP0289 and the intergenic region between them. Pivi77 likely regulates the vacA paralogue encoded by HP0289.

Figure 5. The ΔcagZ H. pylori mutant out-competes the wild-type strain in a co-infection colonization assay. Single strain infection and co-infection studies for H. pylori ΔcagZ and wild-type strains were carried out in FVB/N mice, for two weeks as described in the methods. One representative set of experiments is shown. Each point represents one mouse stomach, and the solid line represents the average. When the ΔcagZ mutant strain was co-infected with the wild-type strain, we were unable to detect
any wild-type strain, and thus placed it at our limit of detection of 200 CFU/gm. This
suggests the ∆cagZ mutant strain out-competed the wild-type strain for colonization
(competitive index (CI) = 5,713).

Figure 6. The ∆mobA,B,D H. pylori mutant is defective for host colonization. Single
strain infection and co-infection studies for H. pylori ∆mobABD and wild-type strains
were carried out in FVB/N mice for two weeks. Each point represents one mouse
stomach, and the solid line represents the average. The ∆mobABD was not recovered
from stomach platings of mice co-infected with both the ∆mobABD mutant and wild-type
strains indicating it was out-competed by the wild-type strain (competitive index (CI) =
0.007).
Figure 1

A. 1) 

2) resl \(\rightarrow\) \(Km^R\) \(\rightarrow\) resl

B. 1) 

2) resl \(\rightarrow\) \(Km^R\) \(\rightarrow\) resl

3) resl \(\xrightarrow{P}\) TnpR

\(Km^S\)
Figure 2

A. Sau3A digested *H. pylori* genomic DNA

B. *H. pylori* chromosome

C. *Km^R^* *H. pylori* RIVET strains

D. Isolate *H. pylori* from stomachs

Km\(^+\) media

Replica plate

Km\(^s\) *H. pylori*
Figure 3
Figure 5

CFU/gm stomach

† cagZ

Δ cagZ

single strain infection

co-infection

ACCEPTED
Figure 6

The graph shows the comparison of CFU/gm stomach between different infection conditions and strains. The x-axis represents the infection type: single strain infection and co-infection, while the y-axis represents the CFU/gm stomach levels ranging from $1 \times 10^2$ to $1 \times 10^7$. The strains include wild type and Δmob, with data points indicating the CFU counts for each condition.
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*

Volume 76, no. 12, p. 5632–5644, 2008. Page 5638: Figure 4 should appear as shown here.

![Diagram of gene expression](image)

**FIG. 4.**

Page 5638: The legend to Fig. 4B should read as follows. “(B) *Pivi51* is 95 bp in length and overlaps *mesJ* and the intergenic region upstream of *mesJ*. However, since *Pivi51* is antisense to *mesJ*, it does not regulate *mesJ*, but rather an as-yet-unidentified transcript.”

Page 5638, column 2: Lines 33–44 should read as follows. “*Pivi51* regulates an as-yet-unidentified open reading frame. *Pivi51* overlaps HP1182 and the region upstream of HP1182. However, *Pivi51* is located on the reverse strand and thus is not oriented correctly to direct expression of HP1182. It is not immediately obvious which open reading frame is regulated by *Pivi51*.”