

# Development of a Tetracycline-Inducible Gene Expression System for the Study of *Helicobacter pylori* Pathogenesis

Aleksandra W. Debowski, Phebe Verbrugghe, Miriam Sehnal, Barry James Marshall, Mohammed Benghezal

Ondek Pty. Ltd. and Helicobacter pylori Research Laboratory, School of Pathology and Laboratory Medicine, Marshall Centre for Infectious Disease Research and Training, University of Western Australia, Perth, Western Australia, Australia

Deletion mutants and animal models have been instrumental in the study of *Helicobacter pylori* pathogenesis. Conditional mutants, however, would enable the study of the temporal gene requirement during *H. pylori* colonization and chronic infection. To achieve this goal, we adapted the *Escherichia coli* Tn10-derived tetracycline-inducible expression system for use in *H. pylori*. The *ureA* promoter was modified by inserting one or two *tet* operators to generate tetracycline-responsive promoters, named *uPtetO*, and these promoters were then fused to the reporter *gfpmut2* and inserted into different loci. The expression of the tetracycline repressor (*tetR*) was placed under the control of one of three promoters and inserted into the chromosome. Conditional expression of green fluorescent protein (GFP) in strains harboring *tetR* and *uPtetO-GFP* was characterized by measuring GFP activity and by immunoblotting. The two *tet*-responsive *uPtetO* promoters differ in strength, and induction of these promoters was inducer concentration and time dependent, with maximum expression achieved after induction for 8 to 16 h. Furthermore, the chromosomal location of the *uPtetO-GFP* construct and the nature of the promoter driving expression of *tetR* influenced the strength of the *uPtetO* promoters upon induction. Integration of *uPtetO-GFP* and *tetR* constructs at different genomic loci was stable *in vivo* and did not affect colonization. Finally, we demonstrate tetracycline-dependent induction of GFP expression *in vivo* during chronic infection. These results open new experimental avenues for dissecting *H. pylori* pathogenesis using animal models and for testing the roles of specific genes in colonization of, adaptation to, and persistence in the host.

Deletion mutants in combination with the use of animal models have been instrumental in the study of bacterial pathogenesis. However, the use of gene deletion limits the study to loss-of-function knockout mutants, and this approach runs the risk of selecting for mutants that have adapted to the deletion genotype through secondary mutations. Furthermore, this technique does not allow for investigating whether a specific gene is necessary to maintain the infection after the initial colonization step or whether it is necessary for the entire infection cycle. In an effort to overcome these limitations, conditional knockouts, based on inducible expression of the target gene, have been developed, which are better suited to studying the temporal requirement of specific genes during infection and in physiological settings. These types of knockouts have been used with great success in human pathogens to elucidate mechanisms of disease development and progression for latent tuberculosis (1) and primary pneumonic plague (2).

One bacterium that has not had the same advancement in the development of genetic tools to facilitate learning about carriage, disease development, and persistence mechanisms is the human pathogen *Helicobacter pylori*. *H. pylori* is an ancient member of the human microbiota that has coevolved with humans to dominate the gastric niche (3–5). Infection by this bacterium causes chronic active gastritis, which may develop into peptic ulceration or, more rarely, gastric adenocarcinoma (6, 7). However, the majority of infected individuals (80 to 90%) carry and transmit *H. pylori* without any symptoms of disease (8, 9). Furthermore, there is mounting epidemiological and recent experimental evidence that suggests that *H. pylori* infection is protective against immune diseases such as childhood asthma, allergic rhinitis, and skin allergies (10–14). The use of conditional mutants is of particular importance for the study of *H. pylori* pathogenesis, as infection is persistent, and

clinical diseases develop after many years of chronic inflammation and epithelial damage.

The lack of suitable genetic systems to control gene expression in *H. pylori* has been a handicap in this area of research. Recently, in an effort to overcome these limitations, a genetic tool for *H. pylori* based on the *lacI<sup>q</sup>-pTac* system of *Escherichia coli* has been developed (15). This system has permitted the generation of conditional *H. pylori* mutants to study the physiological function of essential genes (16, 17). Unfortunately, the use of the *lac* repressor system is limited to *in vitro* studies, as the concentrations of the inducer molecule required to regulate this system make it impractical for studies involving animal models (15, 18).

One system that displays the regulatory properties required to control inducible gene expression during infection is the Tet repressor system from *E. coli* (19). This system is well characterized at the molecular level and has become a broadly applied tool in molecular genetics (18, 20). *tet* regulation is based on the Tet repressor (TetR) proteins, which regulate the expression of a family of tetracycline (Tc)-exporting proteins (18, 19). In the absence of Tc, TetR tightly binds the *tet* operators (*tetO*) within the *tet* pro-

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Address correspondence to Aleksandra W. Debowski, [aleksandra.debowski@uwa.edu.au](mailto:aleksandra.debowski@uwa.edu.au), or Mohammed Benghezal, [mbenghezal@ondek.com](mailto:mbenghezal@ondek.com).

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motor and suppresses transcription of the TetR-controlled gene. When Tc enters the cell, it binds TetR and induces a conformational change that results in dissociation of TetR from *tetO* and thus induces the expression of the TetR-controlled gene. Tc can cross biological membranes by diffusion, enabling these inducers to penetrate most bacterial and eukaryotic cells, and the high inducer affinity (nanomolar range) that TetR has for Tc allows induction to occur prior to inhibition of the ribosome (18). In addition, *tet* regulation provides very tight control and sensitive induction, and consequently, efficient *tet*-regulatory systems have been developed for eukaryotes as well as several Gram-positive and Gram-negative bacteria (21–23). Of interest is that *tet* regulation has also facilitated the generation of conditional bacterial mutants, which have been successfully used to study their pathogenesis in mouse infection models of *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Yersinia pestis* (1, 2, 24, 25). To date, however, the development of such a strategy for *H. pylori* is lagging behind, even though such a genetic tool would facilitate the study of *H. pylori* persistence and aid in the elucidation of the different roles that specific genes play in *H. pylori* pathogenesis, genetic adaptation to the host during acute and chronic infection, and disease progression. Here we present the development of the Tn10-derived *tet*-regulatory system from *E. coli* for use in *H. pylori* and demonstrate tetracycline-dependent gene expression in *H. pylori* both *in vitro* and *in vivo* during persistent infection in mice. The use of *tet* regulation for studying *H. pylori* infection in the context of an intact gastric environment will greatly help to improve our understanding of the role that this disappearing human microbe has in human health and disease (26).

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The plasmids and *H. pylori* strains used in this study are listed in Tables S1 and S2 in the supplemental material. *H. pylori* X47 (27) strains were grown at 37°C under microaerobic conditions on Columbia blood agar (CBA) plates containing 5% horse blood and Dent's antibiotic supplement (Oxoid). The use of the counterselectable streptomycin susceptibility (*rpsL*-based) system requires a host strain that possesses a streptomycin-resistant phenotype. The *H. pylori* X47 host strain is a naturally streptomycin-resistant strain (28), and no modifications to this strain were required. When appropriate, antibiotic selection in *H. pylori* was carried out by supplementing medium with chloramphenicol or streptomycin at a final concentration of 10 µg/ml. For *H. pylori* liquid culture, bacteria were grown in brain heart infusion (BHI) medium supplemented with 10% newborn calf serum (NCS) and Dent's antibiotic supplement (Oxoid). Cultures were inoculated with bacteria suspended in phosphate-buffered saline (PBS) to give a starting optical density at 600 nm (OD<sub>600</sub>) of 0.05 and were grown under microaerobic conditions at 37°C and 120 rpm. Growth studies were performed without any prior adaptation of *H. pylori* strains to liquid media. *Escherichia coli* DH5α was grown in Luria-Bertani broth. When necessary, antibiotics were added at the following final concentrations: 100 µg/ml for ampicillin and 20 µg/ml for chloramphenicol. Primers used in this study are listed in Table S3 in the supplemental material.

**Construction of cloning vectors pGltD, pTrpA, and pMdaB.** A 1-kb fragment of the C-terminal end of HP0380 was amplified with primers GltD1 and GltD2, and 1 kb of HP0379 was amplified with primers GltD3 and GltD4. These two fragments were joined together by strand overlapping extension (SOE) PCR (29), using primers GltD1 and GltD4, to generate a 2-kb PCR product containing a multiple-cloning site (MCS) inserted between HP0380 and HP0379 and flanked by *Cla*I and *Sac*II restriction sites. This fragment was cloned into the vector backbone of pBlu-SK-alt [*Xho*I and *Sal*I sites in pBluescript SK(–) were deleted by restriction enzyme digestion and religation] to give pGltD (see Fig. S1 in

the supplemental material). This cloning vector was used to generate suicide plasmids for the insertion of cloned DNA sequences into the *H. pylori* genome between HP0380 and HP0379. Using the same strategy, cloning vector pTrpA was made by using primers TrpA1 through TrpA4 (see Fig. S2 in the supplemental material). pTrpA was used to generate suicide plasmids to insert DNA sequences of interest into the center of the HP1277 open reading frame. Similarly, pMdaB was made by using primers MdaB1 through MdaB4; however, the 2-kb SOE PCR product was cloned into pBlu-SK-alt using *Cla*I and *Not*I restriction sites. pMdaB was used to generate suicide plasmids for the insertion of cloned DNA sequences between HP0630 and HP0631 (see Fig. S3 in the supplemental material).

**Suicide plasmid constructs for generating X47 recipient strains.** Counterselection based on the *rpsL* streptomycin susceptibility determinant (28) was used to introduce *ptetR* and *uPtetO-GFP* constructs into the *H. pylori* chromosome by natural transformation and homologous recombination. Plasmid pTrpA-RCAT was previously used (30); however, its full construction is described here. A *Bam*HI fragment containing the counterselection cassette *rpsL-cat* (31) was cloned into pGltD, pTrpA, and pMdaB to generate pGltD-RCAT, pTrpA-RCAT, and pMdaB-RCAT, respectively. These plasmid constructs were used in natural transformation of naturally streptomycin-resistant wild-type strain X47 to generate the Str<sup>s</sup> and Cm<sup>r</sup> X47 *gltD::rpsL-cat*, X47 *trpA::rpsL-cat*, and X47 *mdaB::rpsL-cat* recipient strains, which were used in subsequent transformations to introduce DNA sequences of interest into the target locus.

**Construction of *H. pylori* strains expressing TetR.** Three *H. pylori* promoters were used to generate different promoter-*tetR* constructs to drive constitutive expression of TetR in *H. pylori*. Constructs *ptetR2* and *ptetR4* were generated by SOE PCR. Briefly, for *ptetR2*, the *amiE* promoter was amplified from strain 26695 genomic DNA by using primers tet1 and tet2, and *tetR* was amplified from pWH1925 BD (32) with primers tet3 and tet9. These two DNA fragments were then joined together by SOE PCR and amplified by using primers tet4 and tet10. For *ptetR4*, the *flaA* promoter was used to drive expression of *tetR* and was generated by using primers tet5 through tet10. A different strategy was used to generate the mutated core urease promoter-*tetR* fusion *ptetR6*. Three sequential rounds of PCR, using three long forward primers and one short reverse primer, were performed to fuse P<sub>taTaat</sub> to *tetR* in a stepwise manner: long forward primer tet11 with reverse primer tet9 (step 1), forward primer tet12 with primer tet9 (step 2), and forward primer tet14 with reverse primer tet10 (step 3). Primers mbtE and mbtE<sup>r</sup> were used to amplify and clone all three *ptetR* constructs into the *Sbf*I and *Eco*RI restriction sites of pMdaB to generate suicide plasmids pMdaB-*ptetR2*, pMdaB-*ptetR4*, and pMdaB-*ptetR6* (see Fig. S4 in the supplemental material). Natural transformation of the *H. pylori* X47 *mdaB::rpsL-cat* recipient strain with these plasmids was performed to generate the X47 *mdaB::ptetR2*, *mdaB::ptetR4*, and *mdaB::ptetR6* strains harboring *ptetR* at the *mdaB* locus. Chromosomal DNA of the resulting streptomycin-resistant transformants was checked for the correct allelic insertion.

**Construction of tetracycline-responsive promoter *uPtetO* with the green fluorescent protein (GFP) gene as the reporter gene.** A 3-step PCR methodology similar to that used to make *ptetR6* was used to make the *uPtetO1-GFP* and *uPtetO2-GFP* constructs. Briefly, *gfpmut2* was amplified from pONDG (33), using primers tetOGFP1 and tetOGFP5 (step 1). In step 2, forward primers tetOGFP2 and tetOGFP3 were used with reverse primer tetOGFP5 for the constructs *uPtetO1-GFP* and *uPtetO2-GFP*, respectively, and finally, in step 3, primers tetOGFP5 and tetOGFP6 were used to complete the *uPtetO* promoters. The final *uPtetO-GFP* constructs contained a modified core mutant urease promoter (containing either one or two *tetO* binding sites) fused to *gfpmut2*, separated by an *Nde*I-cut site and flanked on both ends by several unique restriction sites. Each construct was digested with *Sal*I and *Xba*I and cloned into similarly digested vectors pGltD and pTrpA to generate suicide plasmids pGltD-*uPtetO1-GFP* and pGltD-*uPtetO2-GFP* and pTrpA-*uPtetO1-GFP* and

pTrpA-uPtetO2-GFP, respectively (see Fig. S5 in the supplemental material).

X47 wild-type and X47 *mdaB::ptetR2*, -4, and -6 strains were transformed with pTrpA-RCAT or pGltD-RCAT to generate the respective recipient strains, and natural transformation of these strains with the appropriate suicide vector was performed to replace the *rpsL-cat* counterselectable maker with *uPtetO-GFP* to create a panel of X47 strains expressing both GFP and TetR (OND2036 to OND2063) or GFP alone (OND1991 to OND1993 and OND2092).

**GFP reporter assays.** For the disc diffusion assay, bacteria were plated onto CBA plates and incubated for 14 h at 37°C. Blank discs were placed onto the bacterial lawn, inoculated with 30  $\mu$ l of an anhydrotetracycline (ATc) solution, and incubated for another 24 h before visualization of GFP expression (LAS 3000; Fujifilm). For liquid culture, 5-ml cultures were grown for 14 h to mid-log phase and then induced with 200 ng/ml ATc. After 24 h, bacteria were harvested by centrifugation, washed twice with PBS, and resuspended to an OD<sub>600</sub> of 2. Next, 0.1 ml of the bacterial suspension was transferred into black 96-well plates, and fluorescence at 520 nm after excitation at 485 nm was measured by using a POLARstar Omega plate reader. Single-colony isolates from three independent transformations were analyzed in triplicate, and the data were combined. Fluorescence intensities were normalized to the cell density and expressed as relative fluorescence units (RFU). Wild-type strain X47 was used to measure background fluorescence of the cells.

**Time course experiments of *tet* regulation.** *H. pylori* cultures were grown to mid-log phase in 10 ml of BHI medium. An aliquot of 2 ml was taken from each culture and used for time zero, and an 8-ml aliquot of fresh medium containing 400 ng/ml ATc was added to each culture to give a final volume of 16 ml and 200 ng/ml ATc. The bacteria were incubated for another 30 h, with aliquots taken from each culture at 2, 4, 8, 16, 24, and 30 h after induction with ATc. Bacterial cells were collected by centrifugation and washed twice with PBS before being processed for immunoblot analysis.

**SDS-PAGE and Western blot analysis.** Bacterial whole-cell lysates were prepared by resuspending washed bacteria in ice-cold Tris lysis buffer (50 mM Tris [pH 7.0], 250 mM NaCl, 1.0% [vol/vol] Triton X-100, 100  $\mu$ M phenylmethylsulfonyl fluoride [PMSF]). Cells were incubated on ice for 15 min and then sonicated for 10 s. Insoluble cell debris was removed by centrifugation, and the protein concentration of the clarified supernatant was measured. Equal amounts of protein for each sample were mixed with 3 $\times$  SDS-PAGE sample loading buffer and incubated at 95°C for 10 min. The proteins were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (0.22- $\mu$ m Immobilon membrane; Millipore) at 4°C with a constant voltage of 90 V in transfer buffer (192 mM glycine, 25 mM Tris, 20% [vol/vol] methanol) for 2 h. The membrane was blocked by using 2% bovine serum albumin (BSA) (Sigma) in PBST (PBS [pH 7.4], 0.1% [vol/vol] Tween 20) and then incubated with the appropriate primary antibody, washed, and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. The membrane was washed again, and detection of the secondary HRP conjugate was accomplished by using chemiluminescence (Sigma). For detection of TetR, rabbit polyclonal IgG anti-TetR (MoBiTec) at a dilution of 1:2,000 was used as the primary antibody. For detection of GFP, rabbit polyclonal anti-GFP (Ondek) was used at a dilution of 1:2,000. Mouse anti-rabbit-HRP and rabbit anti-mouse-HRP (Jackson ImmunoResearch Laboratories) secondary antibodies were used at a dilution of 1:10,000. Chemiluminescence was detected by using a LAS 3000 instrument (Fujifilm) (with LAS 3000 V2.2 image reader software).

**Animal experiments.** Mouse procedures were reviewed and approved by the Institutional Animal Care and Animal Ethics Committee of the University of Western Australia. Six- to seven-week-old C57BL/6J female mice were challenged once by oral gavage with 200  $\mu$ l of  $1 \times 10^9$  CFU/ml bacteria. Mice were sacrificed at the indicated time points, and stomachs were removed and homogenized in 1 ml BHI medium by using a tissue lyser (Retch). Homogenates were serially diluted and plated onto *H. pylori*

selective plates (CBA containing 5% horse blood, Dent's antibiotic supplement, polymyxin B at 2,500 U/liter or 0.2975 mg/liter, nalidixic acid at 10 mg/liter, and bacitracin at 100 mg/liter) to determine the bacterial burden. Reisolated clones were assayed for *tet*-responsive GFP expression.

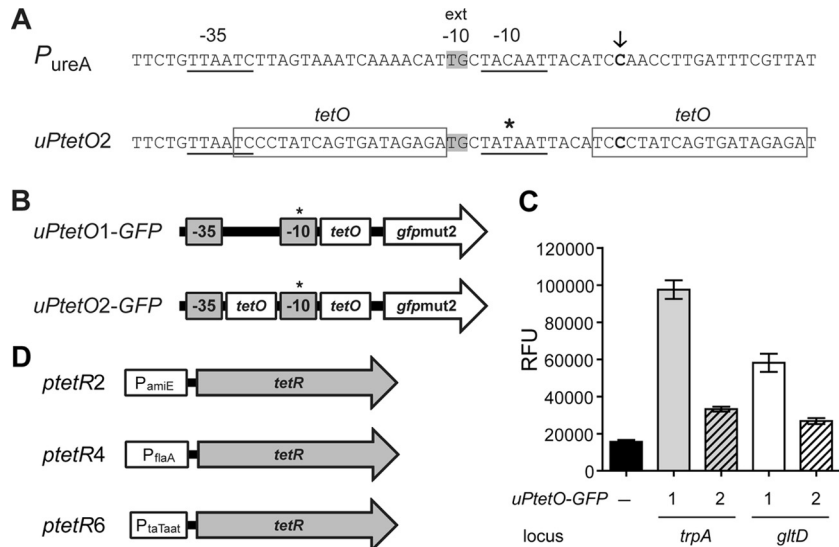
For *in vivo tet* regulation studies, mice were challenged ( $n = 3$  per group) with the conditional GFP-expressing X47 *mdaB::ptetR4 trpA::uPtetO1-GFP* strain (OND2050). Two weeks after challenge, animals were given 20  $\mu$ g/ml ATc or not in their drinking water containing 5% sucrose. Water was kept in light-protected bottles and changed every 3 days. The animals were sacrificed after receiving 4 days of ATc supplementation. Stomachs were removed, opened along the greater curvature, and gently rinsed with PBS to remove stomach contents. Tissue was immediately fixed for 1 h by using 4% paraformaldehyde in PBS. After being embedded in OCT medium and frozen in liquid nitrogen, tissues were cut into 16- $\mu$ m-thick sections.

**Microscopy.** Cryosections were permeabilized with 0.2% Triton X-100, and nonspecific binding sites were blocked with 4% fetal calf serum (FCS) in PBS. Chicken anti-GFP (1:2,000; Abcam) and rabbit anti-*Helicobacter* (1:50; Dakocytomation) primary antibodies were applied for 1 h, followed by incubation of a mixture of goat anti-chicken IgG Alexa Fluor 488 (1:500; Molecular probes) and goat anti-rabbit IgG Dylight 549 (1:500; Jackson ImmunoResearch) secondary antibodies for 30 min. Finally, nuclei were counterstained with Hoechst 333421 (Sigma-Aldrich). Images were collected by using a Nikon Ti-E inverted motorized microscope with a Nikon A1S1 spectral detector confocal system running NIS-C Elements software.

## RESULTS

**Construction and characterization of *tet*-responsive promoters for *H. pylori*.** Previous work with *Mycobacterium*, *Bacillus subtilis*, and *Borrelia burgdorferi* demonstrated that translation of the *tet*-regulatory system from *E. coli* to new bacterial hosts requires the development of *tet*-responsive promoters that are functional in the target bacterium (21, 34–36). Successful strategies have involved replacing nonessential sequences within a strong host promoter with one or more *tetO* sequences. One *H. pylori* promoter that has been extensively characterized is the strong *ureA* promoter P<sub>ureA</sub>, which drives expression of the UreA and UreB subunits of the abundant urease enzyme (37–39). Of interest was that a minimal mutated version of the urease promoter, named P<sub>taTaat</sub>, retained strong basal levels of urease expression without the transcription-regulatory sequences of P<sub>ureA</sub> (38). To avoid any additional regulation in response to fluctuations in environmental pH and free Ni<sup>2+</sup> concentrations (37, 40), P<sub>taTaat</sub> was chosen as a template for developing a *H. pylori tet*-responsive promoter.

Nonessential sequences within P<sub>taTaat</sub> were replaced with one or two *tetO* sequences to generate promoters that could bind TetR (Fig. 1A). These putative *tet*-responsive derivatives of P<sub>taTaat</sub> along with the downstream *ureA* 5' untranslated region (UTR) were designated *uPtetO* constructs (Fig. 1B). Useful reporters that have been used to characterize promoter activity in *H. pylori* are *gfpmut2* and *gfpmut3*, which display very high fluorescence and good folding capacities in bacteria (41–44). Therefore, we chose the reporter *gfpmut2* to test the ability of the two *uPtetO* constructs to drive and regulate gene expression in *H. pylori*. Constructs consisting of *uPtetO* and *gfpmut2*, *uPtetO1-GFP* and *uPtetO2-GFP*, respectively, were generated by using long primers and successive rounds of PCR. The strength of each *uPtetO* promoter was evaluated at two different loci within the *H. pylori* chromosome. The *uPtetO-GFP* constructs were inserted either between HP0379 and HP0380 (*gltD*) or into HP1277 (*trpA*) by using a natural transformation strategy based on the *rpsL-cat* counter-



**FIG 1** Tetracycline-responsive *uPtetO* promoters. (A) Nucleotide sequences of the wild-type *ureA* promoter  $P_{ureA}$  and the tetracycline-responsive promoter  $P_{taTaat}$  derivative *uPtetO2*. The  $-10$  and  $-35$  promoter sequences are underlined, and the extended  $-10$  region is shaded in gray. *tetO* operator (*tetO*) sequences are indicated by boxes. The arrow indicates the transcriptional start point, and the asterisk indicates the C-to-T mutation in the  $-10$  region found in the *uPtetO* promoter constructs. (B) Representative diagram of the *uPtetO* constructs. White *tetO* boxes indicate where  $P_{taTaat}$  promoter sequences have been replaced with *tetO* sequences. (C) GFP activities of *uPtetO1-GFP* (open bars) and *uPtetO2-GFP* (hatched bars) strains compared to wild-type autofluorescence (black bar). Bacteria were transformed as indicated and grown in BHI medium to mid-log phase ( $OD_{600} = 0.5$ ), and GFP activities were determined 24 h later. The fluorescence intensity was normalized to the cell density and expressed as relative fluorescence units (RFU). Data are averages, with error bars representing standard deviations, for three independent clones and are representative of three independent experiments. (D) Schematic diagram of promoter-*tetR* constructs used in this study.

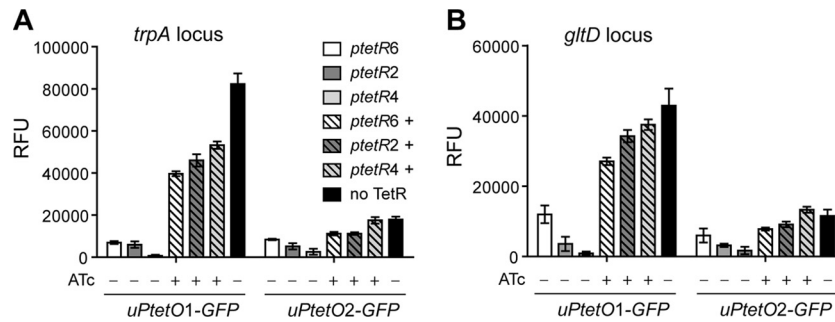
selection cassette (28, 31). These two chromosomal loci were selected because, in our hands, we have found them to have good transformation efficiencies, and their mutagenesis does not affect colonization of strain X47 in the C57BL/6J mouse infection model (data not shown).

GFP activity was quantified in *H. pylori* strains transformed with *uPtetO-GFP* cultivated in liquid medium for 36 h. A comparison of the GFP fluorescence intensities demonstrated that replacement of promoter sequences between the  $-10$  and  $-35$  regions with a second *tetO* site in *uPtetO2* significantly affected its activity compared to *uPtetO1*, as GFP activity was 3- to 4.5-fold weaker when expressed under the control of *uPtetO2* than when expressed under the control of *uPtetO1* (Fig. 1C). The strength of each promoter was also influenced by the sequence context, as the genomic locus into which the constructs were transformed influenced the GFP expression level (Fig. 1C). This locus-dependent effect was more evident for *uPtetO1* than for *uPtetO2*. X47 strains transformed with *uPtetO1-GFP* into the *trpA* locus had 2-fold-higher GFP activity than strains with *uPtetO1-GFP* inserted into the *gltD* locus, while strains transformed with *uPtetO2-GFP* into the *trpA* locus had 1.5-fold-higher GFP activity than strains with *uPtetO2-GFP* inserted into the *gltD* loci. These results demonstrated that the *tetO*-modified  $P_{taTaat}$  promoters could be used to drive expression of foreign genes from the different chromosomal loci.

**Expression of TetR in *H. pylori*.** With functional promoters in hand, we then turned our attention to tetracycline-mediated regulation of the *uPtetO* promoters. Several studies utilizing the *tet* system have shown that constitutive expression of TetR is more favorable for the tight repression of strong promoters than the original autoregulated expression approach derived from the

*Tn10* Tc resistance determinant (23, 34, 45). Therefore, three different *H. pylori* promoters,  $P_{amiE}$ ,  $P_{flaA}$ , and  $P_{taTaat}$ , were selected to drive *tetR* expression (corresponding to constructs *ptetR2*, *ptetR4*, and *ptetR6*, respectively [Fig. 1D]), in order to provide a range of TetR expression levels and permit the fine-tuning of gene induction under different growth conditions. The *ptetR* constructs were generated by SOE PCR and cloned into suicide vector pMdaB to introduce them by natural transformation into the *H. pylori* chromosome between HP0630 and HP0631 (*mdaB* locus). TetR-positive strains were subsequently transformed with different *uPtetO-GFP* constructs to generate a set of strains (OND2036 to OND2063) that constitutively expressed TetR and expressed GFP under the control of a *tetO*-containing promoter.

**Induction of *uPtetO* by ATc.** Anhydrotetracycline (ATc), a less toxic derivative of Tc with very high affinity for *Tn10* TetR (46), was used as an inducer of TetR, and GFP activity was used as a reporter to measure the induction and repression potential of *tet* promoters *uPtetO1* and *uPtetO2*. Under standard growth conditions, these strains had significantly reduced GFP activities compared to those of strains lacking TetR, demonstrating that TetR efficiently repressed gene expression from both *uPtetO* constructs (Fig. 2). Addition of 200 ng/ml ATc to TetR-expressing strains grown in BHI medium resulted in an increase in GFP activity after 24 h; however, GFP activity did not reach the levels observed in the absence of TetR (Fig. 2). After 24 h, induction of *uPtetO1* ranged between 2- and 80-fold, while induction of *uPtetO2* in TetR-expressing strains was much weaker and ranged from 1.3- to 8-fold (Table 1). The X47 *mdaB::ptetR4 trpA::uPtetO1-GFP* strain (OND2050) had the greatest induction range of all the strains tested, displaying an 80-fold increase in GFP activity upon addition of ATc.



**FIG 2** TetR-controlled gene expression in *H. pylori*. Shown is a comparison of *uPtetO1* and *uPtetO2* induction in strains with different *ptetR* constructs. Strains expressing TetR were transformed with the *tet*-responsive constructs *uPtetO1-GFP* and *uPtetO2-GFP* into either the *trpA* (A) or *gltD* (B) locus. Bacteria were transformed as indicated and grown in BHI medium to mid-log phase ( $OD_{600} = 0.5$ ) before 200 ng/ml ATc was added (hatched bars). GFP activities were determined 24 h later. Three independently generated clones were used to measure GFP activities for each strain. All fluorescence measurements were carried out in triplicate. The fluorescence intensity was normalized to the cell density and is expressed as RFU. All fluorescence intensities were corrected for background fluorescence of the bacteria. Data are averages, with error bars representing standard deviations, for three independent clones and are representative of two independent experiments.

**Induction of *uPtetO* by ATc in *H. pylori* is dose and time dependent.** The *tet* system in *H. pylori* was further characterized by measuring GFP activity after induction with increasing inducer concentrations and by evaluating reporter expression at different time points. In addition, a disc diffusion assay was used to demonstrate ATc-dependent gene regulation on solid media. Discs inoculated with different concentrations of ATc were placed onto a bacterial lawn of the X47 *mdaB::ptetR4 trpA::uPtetO1-GFP* strain (OND2050) (Fig. 3A). GFP expression was evident after 24 h and was confined to the regions around each disc where the ATc concentration was sufficient to induce *uPtetO1*.

Quantification of GFP activities of *H. pylori* strains grown in the presence of different concentrations of ATc for 24 h demonstrated that induction of promoters *uPtetO1* and *uPtetO2* was dependent on the inducer concentration (Fig. 3B). Maximal induction was achieved in all strains with the *uPtetO1-GFP* construct at a concentration of 100 ng/ml ATc (Fig. 3B), which, importantly, is a concentration 10-fold below the MIC for this compound, as measured in liquid culture (Fig. 3C). Slightly higher concentra-

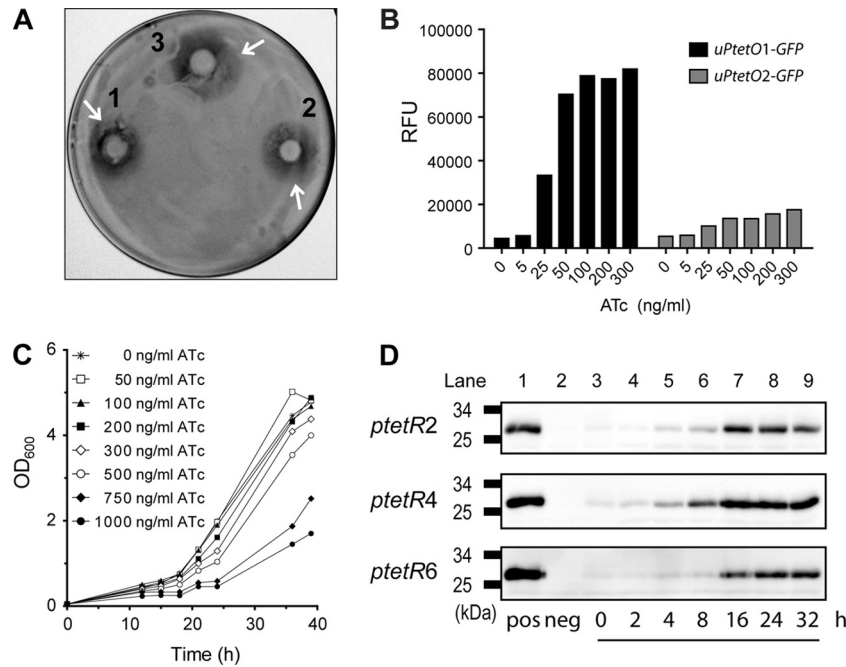
tions of ATc were required to reach maximal GFP activities for some strains with the *uPtetO2-GFP* construct (Fig. 3B).

The kinetics of *uPtetO1-GFP* induction was also analyzed by immunoblotting against GFP (Fig. 3D). GFP expression increased with ATc incubation time and reached a maximum signal 16 h after the addition of ATc. Strains expressing *tetR* under the control of the *flaA* promoter (*ptetR4*) had the greatest range in GFP expression, responding to 5 ng/ml ATc and reaching maximal levels of GFP sooner than strains that were transformed with *ptetR2* or *ptetR6*. Conversely, in strains expressing the largest amount of TetR, *ptetR6* (see Fig. S6 in the supplemental material), induction of *uPtetO* was less complete and required more time; however, repression of *uPtetO* in the absence of ATc was not more efficient. The *ptetR*-dependent effect on *uPtetO2* regulation was not as pronounced in *uPtetO2-GFP*-transformed strains due to the smaller range in expression levels between induced and repressed states. Addition of the second *tetO* site did not result in measurable improvement in silencing of *uPtetO2* compared to *uPtetO1* (Fig. 2 and 3B).

***tet* regulation of *uPtetO* in vivo.** The results obtained from *in vitro* induction experiments demonstrated that *tet*-regulated gene expression in *H. pylori* was functional and thus could serve as a useful tool to study *H. pylori* virulence factors. Therefore, the potential for *tet* regulation of *H. pylori* genes during infection was investigated. The first step was to assess the expression stability of the GFP reporter gene under *tet* regulation after *in vivo* passage. Mice were orally challenged once with X47 *mdaB::ptetR4* recipient strains transformed with either pGltD-*uPtetO1-GFP* or pTrpA-*uPtetO1-GFP*. Colonies were successfully reisolated 1 week after oral challenge (see Fig. S7 in the supplemental material), and GFP expression of reisolated strains remained ATc dependent, demonstrating the genetic stability of the chromosomally integrated *tet*-inducible system in *H. pylori*. With this result in hand, ATc-dependent regulation of GFP expression during *H. pylori* infection was investigated. Two groups of mice ( $n = 3$ ) were challenged with the X47 *mdaB::ptetR4 trpA::uPtetO1-GFP* strain (OND2050), which expressed GFP only in the presence of tetracyclines. Two weeks after challenge, one group of animals was treated with ATc by supplementation in the drinking water, while the other group was maintained on standard

**TABLE 1** Induction and repression of *uPtetO* in *H. pylori*

Strain	<i>ptetR</i> construct	Promoter	Expression locus	Promoter strength (RFU/OD <sub>600</sub> )		Fold increase
				-ATc	+ATc	
OND2038	<i>ptetR2</i>	<i>uPtetO1</i>	<i>trpA</i>	5,939	46,019	8
OND2036			<i>gltD</i>	3,597	34,248	10
OND2039	<i>uPtetO2</i>		<i>trpA</i>	5,146	11,200	2
OND2037			<i>gltD</i>	3,154	9,127	3
OND2050	<i>ptetR4</i>	<i>uPtetO1</i>	<i>trpA</i>	668	53,199	80
OND2048			<i>gltD</i>	885	37,488	42
OND2051	<i>uPtetO2</i>		<i>trpA</i>	2,561	17,495	7
OND2049			<i>gltD</i>	1,683	13,300	8
OND2062	<i>ptetR6</i>	<i>uPtetO1</i>	<i>trpA</i>	6,917	39,628	6
OND2060			<i>gltD</i>	11,985	27,063	2
OND2063	<i>uPtetO2</i>		<i>trpA</i>	8,386	11,167	1.3
OND2061			<i>gltD</i>	5,961	7,802	1.3



**FIG 3** Determination of optimal inducer concentration, growth inhibition by ATc, and kinetics of induction. (A) Regulation of GFP expression on CBA plates. Blank discs were inoculated with 30  $\mu$ l of 0.5  $\mu$ g/ml (1), 1.0  $\mu$ g/ml (2), and 5  $\mu$ g/ml (3) of ATc, placed onto a bacterial lawn of the *mdaB::ptetR4 trpA::uptetO1-GFP* strain (OND2050), and incubated for 24 h. The GFP fluorescence signal appears as a dark signal against the lighter background of the bacterial lawn on the CBA plate. The zone of GFP expression is indicated (white arrows). Discs were removed before the image was taken. (B) Inducer concentration. *H. pylori* strains expressing TetR were transformed with *tet*-responsive construct *uPtetO1-GFP* or *uPtetO2-GFP* and grown in BHI medium to mid-log phase ( $OD_{600} = 0.5$ ) prior to the addition of increasing amounts of ATc. GFP activities of each culture were recorded every 3 h. (C) Growth curves of *H. pylori* X47 grown in BHI medium without or with different concentrations of ATc. The optical densities of each culture were recorded every 3 h. (D) Time course of TetR-controlled GFP expression. Shown is induction of *uPtetO1* in strains transformed with different promoter-*tetR* constructs,  $P_{amiE}$ -*tetR* (*ptetR2*),  $P_{flaA}$ -*tetR* (*ptetR4*), and  $P_{taTaat}$ -*tetR* (*ptetR6*). Bacteria were grown in BHI medium to mid-log phase ( $OD_{600} = 0.5$ ) before the addition of 200 ng/ml of ATc. Aliquots of induced cultures were taken at the indicated time points. *H. pylori* lysates were separated on a 10% SDS-PAGE gel. Equal amounts of protein (15  $\mu$ g) were loaded into each lane. Lane 1, GFP constitutively expressed by X47 lacking TetR (pos); lane 2, wild-type strain X47 (neg); lane 3, repressed GFP (+TetR); lanes 4 to 9, time course of induction of TetR-controlled GFP by 200 ng/ml ATc.

drinking water. Animals were sacrificed 4 days later, and confocal fluorescence imaging of infected mouse stomachs detected both *H. pylori* and GFP when probed with anti-*H. pylori* antibodies and anti-GFP antibodies, respectively (Fig. 4). Importantly, larger amounts of GFP protein were detected in the stomachs of all three mice that received tetracycline supplementation than in the stomach samples from infected mice that remained untreated. Superimposition of *H. pylori* and GFP fluorescent signals led to a dotted pattern suggesting colocalization of GFP with *H. pylori* cells. This confocal data demonstrated that gene expression from the *tet*-responsive *uPtetO1* promoter could indeed be regulated *in vivo* during the persistence stage of infection using low levels of ATc supplementation.

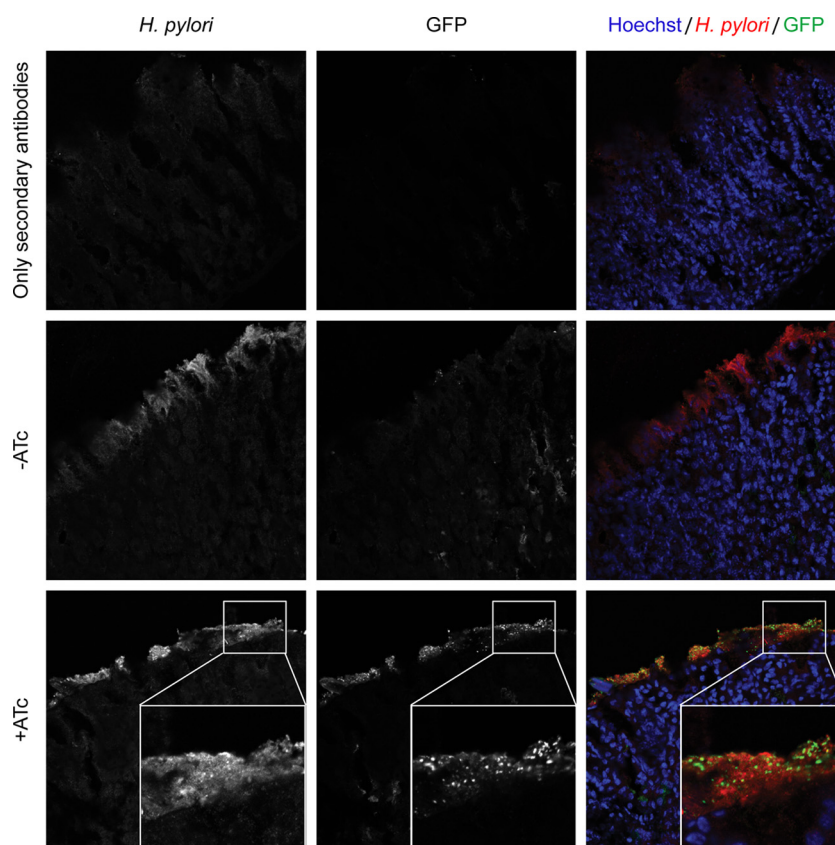
## DISCUSSION

The use of tetracycline-dependent gene regulation has not been previously reported for *H. pylori* or for any other members of the epsilonproteobacteria, including the closely related campylobacters. We developed two *tet*-responsive promoters that are functional in *H. pylori* and characterized their regulation using GFP as a reporter. The regulatory range achieved in this study (up to 80-fold) is comparable to the those of first-generation *tet* regulation systems adapted to other bacteria such as *B. subtilis* (100-

fold), *S. aureus* (50- to 100-fold), *Mycobacterium smegmatis* (170-fold), and *Streptococcus pneumoniae* (5-fold) (21, 24, 34, 47).

A comparison of the activity and regulation of the two promoters revealed that the introduction of a second *tetO* site into *uPtetO2* led to significantly reduced promoter activity (Fig. 1C) although without improvement of TetR-mediated silencing (Fig. 2) compared to *uPtetO1*. The lower activity of *uPtetO2* may be attributed to the decrease in AT content upstream of the -14 position brought about by the introduction of *tetO*, as a recent analysis of ~2,000 transcription start signals identified this periodic AT-rich signal as a moderately conserved feature in *H. pylori* promoters (48).

The availability of *tet* promoters with different strengths allows for the construction of conditional mutants in which *tet*-regulated expression more closely reflects that of the target gene of interest. Further refinements can be achieved through selection of the appropriate recipient locus, as *tet* promoter activity was also shown to be influenced by the genomic locus into which the constructs were transformed. Variations in gene expression due to chromosomal positioning are well documented for other bacteria such as *E. coli* (49), *Salmonella enterica* serovar Typhimurium (50), and *Lactococcus lactis* (51) and have been attributed to the operative increase in gene dosage associated with regions close to *oriC*. Although *H. pylori* is unlikely to replicate multiple genomes like *E.*



**FIG 4** Induction of GFP expression in *H. pylori* during infection. Mice were infected by oral gavage with the conditional GFP-expressing *H. pylori* X47 *mdaB::ptetR4 trpA::uPtetO1-GFP* strain (OND2050). Two weeks after infection, one group of mice received 20  $\mu\text{g/ml}$  ATc for 4 days in their drinking water (top and bottom), whereas the control groups were kept without ATc supplement (middle). The top row depicts sections stained with secondary antibodies only. The left column depicts stomach tissues stained with antibody against *H. pylori*, the middle column depicts tissue stained with antibody against GFP, and the right column shows cell nuclei counterstained with Hoechst (blue), merged with anti-*H. pylori* (red) and anti-GFP (green).

*coli* (30), the observed positional effect on *uPtetO* activity provides an additional mechanism by which gene expression in conditional mutants can be further adjusted to match the levels of gene expression in wild-type strains.

Finally, adjustments to the regulatory windows to suit expression of a target gene were also achieved by adjusting *tetR* expression by way of different promoters. The use of  $P_{\text{taTaat}}$  to drive *tetR* expression resulted in the highest steady-state levels of TetR protein, followed by  $P_{\text{amiE}}$  and then  $P_{\text{flaA}}$ , which produced the smallest amount of TetR. These differences, as detected by immunoblotting, were not very large (see Fig. S6 in the supplemental material); however, they had significant effects on *uPtetO1* regulation (Fig. 3B and D). Smaller amounts of TetR protein have been shown to make the *tet* system more sensitive to the presence of ATc and lead to faster induction responses (19, 52), which was observed in this study for strains transformed with *ptetR4*, as these strains expressing *tetR* under the control of the *flaA* promoter had the greatest range in GFP expression levels.

Interestingly, the response to *tet* regulation in *H. pylori*, as demonstrated by the time course assays, was relatively slow compared to those of other bacterial species (21, 23). This slow-induction profile is in line with the delayed induction described previously for the *lacI<sup>q</sup>* conditional expression system in *H. pylori* (15), and it is likely to reflect intrinsic characteristics of *H. pylori* such as a low growth rate.

Confocal imaging of infected stomachs demonstrated that the activity of the *tet*-responsive *uPtetO* promoter can be regulated during persistent infection by using low levels of tetracycline supplementation. This suggests that *tet* regulation is a very valuable tool to study *H. pylori* pathogenesis and disease progression in established animal models.

In conclusion, this study describes different *uPtetO* and *ptetR* constructs that can be combined with new chromosome positions that are amenable to neutral gene insertion, to provide three different levels of regulation for modulating and fine-tuning gene expression in conditional mutants. *tet*-regulated GFP expression both *in vitro* and *in vivo* has established that *tet*-regulated gene expression in *H. pylori* is possible and has shown that further experimentation with the *tet* system as a genetic tool to study *H. pylori* virulence factors is warranted.

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