Development of a tetracycline-inducible gene expression system for the study of Helicobacter pylori pathogenesis

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

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

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 activity 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 pulmonary 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 lacP–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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moter 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 intact gastric environments (18). In addition, tet regulation provides very tight control and sensitive induction, and consequently, efficient tet-regulatory systems have been developed for eukaryotic 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 adaption 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 (OD600) 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 Clal and SacII restriction sites. This fragment was cloned into the vector backbone of pBlu-SK-alt [Xhol and Sall 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 Clal and NotI 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 plasmids 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 BamHI fragment containing the counterselectable 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 Strr and Cmfr 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 amrE 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 PtaTaat 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 mbtetF and mbtetR were used to amplify and clone all three ptetR constructs into the SfiI and EcoRI 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 uPtet0 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 urease promoter (containing either one or two tetO binding sites) fused to gfpmut2, separated by an NdeI-cut site and flanked on both ends by several unique restriction sites. Each construct was digested with Sall and XbaI and cloned into similarly digested vectors pGltD and pTrpA to generate suicide plasmids pGltD- uPtetO1-GFP and pGltDpUptetO2-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 pGID-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 marker 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 μl 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 unless otherwise stated. After 24 h, bacteria were harvested by centrifugation, washed twice with PBS, and resuspended to an OD600 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 μM 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 3X 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 (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/6 female mice were challenged once by oral gavage with 200 μl of 1 × 10^8 CFU/ml mice. Bacteria 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 wereserially 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 trpAs:: uPtetO1-GFP strain (OND2050). Two weeks after challenge, animals were given 20 μg/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-μm-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; Dako) 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 33342 (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 PureA 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 PtaTaat, retained strong basal levels of urease expression without the transcription-regulatory sequences of PureA (38). To avoid any additional regulation in response to fluctuations in environmental pH and free Ni^2+^ concentrations (37, 40), PtaTaat was chosen as a template for developing a *H. pylori* tet-responsive promoter.

Nonessential sequences within PtaTaat were replaced with one or two tetO sequences to generate promoters that could bind TetR (Fig. 1A). These putative tet-responsive derivatives of PtaTaat 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 (gldD) or into HP1277 (trpA) by using a natural transformation strategy based on the rpsL-cat counter-
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 \textit{H. pylori} strains transformed with \textit{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 \(-10\) region had a 1.5-fold-higher GFP activity than strains with \textit{uPtetO} into the \textit{tetO} site in \textit{uPtetO} significantly affected its activity compared to \textit{uPtetO}, as GFP activity was 3- to 4.5-fold weaker when expressed under the control of \textit{uPtetO} than when expressed under the control of \textit{uPtetO} (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 \textit{uPtetO1} than for \textit{uPtetO2}. X47 strains transformed with \textit{uPtetO1-GFP} into the \textit{trpA} locus had 2-fold-higher GFP activity than strains with \textit{uPtetO1-GFP} inserted into the \textit{gltD} locus, while strains transformed with \textit{uPtetO2-GFP} into the \textit{trpA} locus had 1.5-fold-higher GFP activity than strains with \textit{uPtetO2-GFP} inserted into the \textit{gltD} loci. These results demonstrated that the \textit{tetO}-modified \textit{P\textsubscript{uTaat}} promoters could be used to drive expression of foreign genes from the different chromosomal loci.

Expression of TetR in \textit{H. pylori}. With functional promoters in hand, we then turned our attention to tetracycline-mediated regulation of the \textit{uPtetO} promoters. Several studies utilizing the \textit{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 \textit{Tn10 Tc} resistance determinant (23, 34, 45). Therefore, three different \textit{H. pylori} promoters, \textit{P\textsubscript{ureA}}, \textit{P\textsubscript{flaA}}, and \textit{P\textsubscript{ptaTaat}}, were selected to drive \textit{tetR} expression (corresponding to constructs \textit{ptetR2}, \textit{ptetR4}, and \textit{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 \textit{ptetR} constructs were generated by SOE PCR and cloned into suicide vector pMdB to introduce them by natural transformation into the \textit{H. pylori} chromosome between \textit{HP0630} and \textit{HP0631} (\textit{mdaB} locus). TetR-positive strains were subsequently transformed with different \textit{uPtetO-GFP} constructs to generate a set of strains (OND2036 to OND2063) that constitutively expressed TetR and expressed GFP under the control of a \textit{tetO}-containing promoter.

Induction of \textit{uPtetO} by ATc. Anhydrotetracycline (ATc), a less toxic derivative of Tc with very high affinity for \textit{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 \textit{tet} promoters \textit{uPtetO1} and \textit{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 \textit{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 \textit{uPtetO1} ranged between 2- and 80-fold, while induction of \textit{uPtetO2} in TetR-expressing strains was much weaker and ranged from 1.3- to 8-fold (Table 1). The \textit{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.
Induction of \textit{uPtetO} by ATc in \textit{H. pylori} is dose and time dependent. The \textit{tet} system in \textit{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 \textit{X47 mda\textsubscript{B}: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 \textit{uPtetO1}.

Quantification of GFP activities of \textit{H. pylori} strains grown in the presence of different concentrations of ATc for 24 h demonstrated that induction of promoters \textit{uPtetO1} and \textit{uPtetO2} was dependent on the inducer concentration (Fig. 3B). Maximal induction was achieved in all strains with the \textit{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 concentrations of ATc were required to reach maximal GFP activities for some strains with the \textit{uPtetO2-GFP} construct (Fig. 3B).

The kinetics of \textit{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 \textit{tetR} under the control of the \textit{flaA} promoter (\textit{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 \textit{ptetR2} or \textit{ptetR6}. Conversely, in strains expressing the largest amount of \textit{tetR}, \textit{ptetR6} (see Fig. S6 in the supplemental material), induction of \textit{uPtetO} was less complete and required more time; however, repression of \textit{uPtetO} in the absence of ATc was not more efficient. The \textit{tetR}-dependent effect on \textit{uPtetO} regulation was not as pronounced in \textit{uPtetO2-GFP}-transformed strains due to the smaller range in expression levels between induced and repressed states. Addition of the second \textit{tetO} site did not result in measurable improvement in silencing of \textit{uPtetO2} compared to \textit{uPtetO1} (Fig. 2 and 3B).

\textit{tet} regulation of \textit{uPtetO} in \textit{vivo}. The results obtained from \textit{in vitro} induction experiments demonstrated that \textit{tet}-regulated gene expression in \textit{H. pylori} was functional and thus could serve as a useful tool to study \textit{H. pylori} virulence factors. Therefore, the potential for \textit{tet} regulation of \textit{H. pylori} genes during infection was investigated. The first step was to assess the expression stability of the GFP reporter gene under \textit{tet} regulation after \textit{in vivo} passage. Mice were orally challenged once with \textit{X47 mda\textsubscript{B}:ptetR4} recipient strains transformed with either pGltD\textunderscore uPtetO1-GFP or pTrpA\textunderscore 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 \textit{tet} dependent, demonstrating the genetic stability of the chromosomally integrated \textit{tet}-inducible system in \textit{H. pylori}. With this result in hand, \textit{ATc}-dependent regulation of GFP expression during \textit{H. pylori} infection was investigated. Two groups of mice (n = 3) were challenged with the \textit{X47 mda\textsubscript{B}: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

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Strain} & \textbf{ptetR} & \textbf{Promoter} & \textbf{Expression} & \textbf{Fold} \\
& \textbf{construct} & & \textbf{locus} & \textbf{strength} \\
& & & & \textbf{ATc} / \textbf{ATc} \\
\hline
OND2038 & \textit{ptetR2} & \textit{uPtetO1} & \textit{trpA} & 5,939 / 46,019 & 8 \\
OND2036 & & & \textit{gltD} & 3,597 / 34,248 & 10 \\
OND2039 & \textit{uPtetO2} & \textit{trpA} & & 5,146 / 11,200 & 2 \\
OND2037 & & & \textit{gltD} & 3,154 / 9,127 & 3 \\
OND2050 & \textit{ptetR4} & \textit{uPtetO1} & \textit{trpA} & 668 / 53,199 & 80 \\
OND2048 & & & \textit{gltD} & 885 / 37,488 & 42 \\
OND2051 & \textit{uPtetO2} & \textit{trpA} & & 2,561 / 17,495 & 7 \\
OND2049 & & & \textit{gltD} & 1,683 / 13,300 & 8 \\
OND2062 & \textit{ptetR6} & \textit{uPtetO1} & \textit{trpA} & 6,917 / 39,628 & 6 \\
OND2060 & & & \textit{gltD} & 11,985 / 27,063 & 2 \\
OND2063 & \textit{uPtetO2} & \textit{trpA} & & 8,386 / 11,167 & 1.3 \\
OND2061 & & & \textit{gltD} & 5,961 / 7,802 & 1.3 \\
\hline
\end{tabular}
\caption{Induction and repression of \textit{uPtetO} in \textit{H. pylori}}
\end{table}
Blank discs were inoculated with 30 Gfp by 200 ng/ml ATc. X47 lacking TetR (pos); lane 2, wild-type strain X47 (neg); lane 3, repressed GFP (epsilonproteobacteria, including the closely related campylobac-

tiological in previously reported for

DISCUSSION

Antibodies and anti-GFP antibodies, respectively (Fig. 4). Im-

portantly, larger amounts of GFP protein were detected in the stomachs of all three mice that received tetracycline supple-

mentation 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 colocal-

ization of GFP with H. pylori cells. This confocal data demon-

strated that gene expression from the tet-responsive upTetO1 promoter could indeed be regulated in vivo during the persist-

ence stage of infection using low levels of ATc supplemen-

tation.

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 campylobac-

ters. We developed two tet-responsive promoters that are func-

tional 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 promot-

ers 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 period-

ic 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 ap-

propriate 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 chromo-

somal 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. Al-

though H. pylori is unlikely to replicate multiple genomes like E.

coli (30), the observed positional effect on $uP_{tetO}$ 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 $P_{taTaat}$ to drive $tetR$ expression resulted in the highest steady-state levels of TetR protein, followed by $P_{amiE}$ and then $P_{flaA}$, 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 $uP_{tetO}$ 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 $ptetR$, 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 $lacP^1$ 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 $uP_{tetO}$ 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 $uP_{tetO}$ 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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