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Control of gene expression in *Helicobacter pylori* using the Tet repressor

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Abstract

The lack of a versatile system to control gene expression in *Helicobacter pylori* has hampered efforts to study *H. pylori* physiology and pathogenesis. To overcome these limitations, we evaluated the utility of an inducible system based on the well-characterized Tet repressor (TetR) and Tet operator (*tetO*). As validation of this system, we introduced three copies of *tetO* into the promoter region upstream of the *cagUT* operon (encoding two virulence factors required for function of the *H. pylori* Cag type IV secretion system) and expressed *tetR* by introducing a codon-optimized gene into the chromosomal *ureA* locus. Introduction of the *tetO* copies upstream of *cagUT* did not disrupt promoter activity, as determined by immunoblotting for CagT. The subsequent introduction of *tetR*, however, did repress CagT synthesis. Production of CagT was restored when strains were cultured in the presence of the inducer, anhydrotetracycline. To demonstrate one potential application of this new tool, we analyzed the function of the Cag type IV secretion system. When the modified *H. pylori* strains were co-cultured with AGS cells, activity of the Cag type IV secretion system was dependent on the presence of anhydrotetracycline as evidenced by inducer-dependent induction of IL-8 secretion, CagA translocation, and appearance of type IV secretion system pili at the bacteria-host interface. These studies demonstrate the effectiveness of the *tetR-tetO* system to control gene expression in *H. pylori* and provide an improved system for studying *H. pylori* physiology and pathogenesis.

Keywords

Helicobacter pylori genetics/physiology; Bacterial gene expression/regulation; Bacterial genetic engineering; Genetic promoter regions

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1. Introduction

Helicobacter pylori are Gram-negative bacteria that can establish persistent colonization of the human stomach (Suerbaum and Michetti 2002; Atherton and Blaser 2009; Cover and Blaser 2009). All people infected by *H. pylori* develop chronic superficial gastritis, and colonization of the stomach is considered a risk factor for peptic ulcer disease, gastric lymphoma, and gastric adenocarcinoma (IARC 1994; Yamada, Ahnen et al. 1994; Fox and Wang 2007; Wroblewski, Peek et al. 2010). The risk of the latter diseases is influenced by multiple factors, including host genetics, dietary or environmental factors, and strain-specific variations in *H. pylori*.

Most studies of *H. pylori* gene function have involved the study of mutant strains in which individual genes are disrupted or deleted (Copass, Grandi et al. 1997; Dailidienė, Dailide et al. 2006; Shaffer, Gaddy et al. 2011; Debowski, Gauntlett et al. 2012; Noto and Peek 2012). Such approaches have been important in characterizing a variety of virulence factors, including the *H. pylori* *cag* type IV secretion system (T4SS). This system consists of a multiprotein complex that delivers the CagA oncoprotein and peptidoglycan into epithelial cells (Backert and Selbach 2008). Upon its entry into the host cell cytoplasm, CagA can be phosphorylated by host cell kinases (Odenbreit, Puls et al. 2000; Stein, Rappuoli et al. 2000). CagA, in both phosphorylated and non-phosphorylated forms, is able to interact with host cell signaling proteins, resulting in an assortment of consequences, including cytoskeletal alterations, disruption of cellular junctions, and altered cellular adhesion and polarity (Amieva, Vogelmann et al. 2003; Hatakeyama 2004; Bourzac and Guillemain 2005). Delivery of peptidoglycan into the host cell cytoplasm by the Cag T4SS results in activation of nucleotide-binding oligomerization domain 1 (NOD1), leading to the production of proinflammatory cytokines MIP-2, β -defensin, and IL-8 (Viala, Chaput et al. 2004; Allison, Kufer et al. 2009).

Analysis of mutant strains has provided important insights into various features of the *cag* type IV secretion system, but knock-out or loss-of-function mutants can be blunt instruments. Such mutants do not allow one to determine whether a given gene is required throughout the infectious process or whether the gene is only required to establish or to maintain the infection (Salama, Otto et al. 2001; Liu, Gao et al. 2008; Barrozo, Cooke et al. 2013). Such mutants also are not suited for the study of essential genes (which cannot be disrupted or deleted). An easily regulated system to specifically control expression of a gene of interest could overcome these limitations, and could also provide an alternative approach, instead of comparisons of isogenic mutant and complemented mutant strains, for analyzing the functions of genes.

Previous studies have exploited inducible promoters in *H. pylori*, but the experimental approaches used in these studies have limitations. Multiple studies have used endogenous promoters to regulate gene expression in *H. pylori* (Delany, Pacheco et al. 2001; Ernst, Stoof et al. 2006; Carpenter, McDaniell et al. 2007; Muller, Pflock et al. 2007; Vannini, Agriesti et al. 2012). However, these promoters are regulated by metals (*e.g.*, iron or nickel) known to modulate expression of multiple genes in *H. pylori* (Ernst, Stoof et al. 2006; Whitmire, Gancz et al. 2007; Danielli and Scarlato 2010; Pich and Merrell 2013). Thus, the addition of an inducing metal to *H. pylori* cultures to regulate genes expressed by these promoters complicates analysis of the gene of interest. One previous study overcame this limitation by introducing *lacI^q* on an *E. coli-H. pylori* shuttle plasmid (Boneca, Ecobichon et al. 2008; Thibonnier, Thiberge et al. 2008; Thibonnier, Aubert et al. 2010). However, such shuttle plasmids can be difficult to introduce into some *H. pylori* strains, likely because of restriction barriers between strains.

In the present study, we deployed the well-characterized Tet-Off system, in which transcription of targeted genes is negatively regulated by the tetracycline repressor (TetR), to regulate gene expression in *H. pylori* (Hillen and Wissmann 1989; Gossen and Bujard 1992). In the absence of inducer (e.g., tetracycline), TetR binds to specific operator sequences (*tetO*) located within the promoter region of the regulated gene and represses transcription; addition of inducer disrupts TetR binding to *tetO* and allows transcription. We describe the introduction of a codon-optimized *tetR* as well as copies of the TetR binding site, *tetO*, into the *H. pylori* chromosome, and demonstrate the Tet-dependent production of CagT and multiple phenotypes associated with the Cag T4SS.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

H. pylori strain 26695 was selected for use in this study (Table 1) (Tomb, White et al. 1997). This strain and derivatives developed herein were routinely grown at 37°C on Trypticase soy agar plates containing 5% sheep blood in ambient air containing 5% CO₂. *H. pylori* mutant strains were selected based on resistance to chloramphenicol (2.5 µg ml⁻¹) or resistance to kanamycin (25 µg ml⁻¹) on Brucella agar plates (Brucella broth containing 1.35% agar and 10% fetal bovine serum). *H. pylori* liquid cultures were grown in Brucella broth supplemented with 10% fetal bovine serum (Atlanta Biologicals). Brucella broth and agar media were prepared without addition of sodium bisulfite (Hawrylik, Wasilko et al. 1994). When appropriate, culture media were supplemented with anhydrotetracycline (ATc, 100 ng ml⁻¹).

2.2. Mutagenesis of *cagUT* Promoter and Introduction of *tetR*

To mutate the *cagUT* promoter, a synthetic DNA construct was designed that included about 500 bp of flanking sequence on either side of the identified *cagUT* transcription start site, derived from *H. pylori* strain 26695 (Sharma, Hoffmann et al. 2010; Ta, Hansen et al. 2012). The synthetic construct also included a kanamycin resistance cassette (divergently transcribed relative to *cagUT*) in the intergenic region between *cagU* and *cagV*, and three copies of *tetO* flanking the predicted *cagUT* promoter element (Figure S1). This synthetic construct was cloned into plasmid pUC57 and the resulting plasmid was used to transform *H. pylori* strain 26695, selecting for kanamycin-resistant colonies. PCR and DNA sequencing were used to confirm that homologous recombination via double crossover resulted in the introduction of the engineered *cagV-cagU* intergenic region into the chromosome of *H. pylori*.

A codon-optimized *tetR* gene was synthesized (Genscript; accession number KF697108). To introduce the *tetR* gene into *H. pylori*, we used a plasmid derived from pAD1, which allows genes of interest to be introduced into the *H. pylori* chromosomal *ureA* locus (Loh, Forsyth et al. 2004; Shaffer, Gaddy et al. 2011). The modified pAD1 plasmid contains a chloramphenicol resistance cassette, restriction sites to allow cloning of a gene of interest into a site downstream from the *ureA* promoter and a ribosomal binding site, and flanking sequences derived from the *ureA* locus. A plasmid (pMM682) was constructed to allow expression of a codon-optimized *tetR* sequence from the *ureA* promoter. The appropriate *H. pylori* strain was transformed with this plasmid, and chloramphenicol-resistant colonies were selected. PCR and DNA sequencing were used to confirm that homologous recombination via double crossover resulted in the introduction of *tetR* into the *H. pylori* chromosome.

2.3. Generation of Rabbit Polyclonal anti-CagT Serum

A fragment of CagT derived from *H. pylori* 26695 (amino acids 26–280) was expressed as a GST fusion protein from pGEX-5X-1 vector (GE Healthcare). CagT-GST production was induced with IPTG and the fusion protein was purified using glutathione beads (Busler, Torres et al. 2006). Rabbits were then immunized with purified CagT-GST.

2.4. Immunoblot Analysis

To detect protein production, individual samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and subsequently immunoblotted using rabbit polyclonal antiserum raised against recombinant CagT, rabbit polyclonal anti-CagA (Santa Cruz), mouse monoclonal antibody to TetR (clone 9G9, Clontech), or mouse monoclonal antibody to phosphotyrosine (pY-99, Santa Cruz). Horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG was used as the second antibody. Signals were generated by enhanced chemiluminescence reaction (SuperSignal West Pico Chemiluminescent Substrate, Pierce Chemical) and detection by exposure to X-ray film.

2.5. Cell Culture Methods

AGS human gastric epithelial cells (ATCC) were grown in the presence of 5% CO₂ in RPMI medium containing 10% FBS, 2 mM L-glutamine, and 10 mM HEPES buffer.

2.6. IL-8 Secretion by Gastric Cells in Contact with *H. pylori*

H. pylori strains were co-cultured with AGS cells at a multiplicity of infection of 100:1. IL-8 secretion into the cell culture medium was analyzed using an anti-human IL-8 sandwich ELISA (R&D Systems).

2.7. CagA Translocation Assay

Translocation of CagA into AGS cells was analyzed by co-culturing *H. pylori* strains with AGS cells and detecting tyrosine phosphorylation of CagA, as previously described (Busler, Torres et al. 2006; Loh, Torres et al. 2007). Briefly, *H. pylori* and AGS human gastric cells were co-cultured at a MOI of 100:1 for 7 h at 37°C. Cells were lysed in NP-40 lysis buffer containing Complete Mini EDTA-free Protease Inhibitor (Roche) and Phos-Stop phosphatase inhibitor (Roche), and CagA translocation was assessed by separating the soluble fraction using 7.5% SDS-PAGE and immunoblotting with an anti-phosphotyrosine antibody (α -PY99, Santa Cruz).

2.8. Electron Microscopy of *H. pylori* in Contact with Gastric Epithelial Cells

Imaging of *H. pylori* by field emission scanning electron microscopy was performed essentially as described (Shaffer, Gaddy et al. 2011). *H. pylori* and AGS human gastric cells were co-cultured at a MOI of 100:1 on tissue culture-treated coverslips (BD Biosciences) for 4 h at 37°C in the presence of 5% CO₂. Cells were fixed with 2.0% paraformaldehyde, 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer for 1 h at 37°C. Coverslips were washed with sodium cacodylate buffer and secondary fixation was performed with 1% osmium tetroxide at room temperature for 30 minutes. Coverslips were washed with sodium cacodylate buffer and dehydrated with sequential washes of increasing concentrations of ethanol. Samples were then dried at the critical point, mounted onto sample stubs, grounded with a thin strip of silver paint at the sample edge, and sputter-coated with gold-palladium before viewing with a FEI Quanta 250 FEG scanning electron microscope.

3. Results and Discussion

3.1. Introduction of *tetO* Sites Upstream of *cagUT*

We selected the *cagUT* operon as a potential target to regulate and thereby evaluate the effectiveness of the Tet system in *H. pylori*. This operon has a promoter sequence containing a predicted -10 element upstream of a previously identified transcription start site, appears to be a simple operon with a well-defined start site and does not appear to be complicated by alternate start sites or antisense transcripts (Sharma, Hoffmann et al. 2010; Ta, Hansen et al. 2012). One of the products (CagT) can be monitored by immunoblotting, and the gene products are of interest in the *H. pylori* research community because of their roles in the Cag T4SS (Fischer, Puls et al. 2001; Kutter, Buhrdorf et al. 2008). To regulate expression from the *cagUT* promoter, we first introduced three copies of the *tetO* sequence flanking the *cagUT* promoter into *H. pylori* strain 26695, resulting in strain VM120 (Table 1 and Figures 1 and S1). To determine whether CagT production was disrupted by these alterations in the *cagUT* promoter region, we analyzed CagT levels in strain VM120 and the parental strain 26695 by immunoblotting. Results indicated that the promoter alterations did not disrupt CagT production (Figure 2A).

3.2. TetR-Mediated Repression of Gene Expression

We next introduced a codon-optimized copy of the Tn10 *tetR* gene into the *ureA* locus of strain VM120, generating strain VM122. An immunoblot to detect TetR production and PCR of the chromosomal *ureA* locus were used to confirm construction of the desired strain (Figure 2B and data not shown). The introduction of *tetR* resulted in repression of the *cagUT* operon as evidenced by reduced CagT levels in strain VM122 compared to strains VM120 and VM124 (Figure 2B and data not shown).

3.3. Induction of Gene Expression Using ATc

We next cultured strain VM122 in the absence or presence of the TetR inducer ATc, a derivative of tetracycline that binds the Tet repressor with greater affinity than tetracycline and exhibits markedly reduced antibiotic activity (Degenkolb, Takahashi et al. 1991; Rasmussen, Noller et al. 1991; Oliva, Gordon et al. 1992; Lederer, Kintrop et al. 1996). Cultures then were analyzed for CagT production by immunoblotting. Results indicated that the addition of ATc to the culture medium relieved TetR-mediated repression of the *cagUT* promoter (Figure 2C).

3.4. Inducible Cag T4SS Activity

CagU and CagT are essential to the function of the Cag T4SS, the multiprotein complex that delivers the CagA oncoprotein to gastric epithelial cells (Fischer, Puls et al. 2001; Kutter, Buhrdorf et al. 2008). Although the function of CagU is unknown, CagT is purported to be a part of the “core complex” that spans the bacterial inner and outer membranes and is thus believed to be an important structural component of the secretion apparatus. Using strain VM122, we analyzed Cag T4SS activity and pilus assembly.

As an initial experiment, strain VM122 grown in the absence of inducer was added to AGS cell monolayers in the absence or presence of ATc. One means of assessing activity of the Cag T4SS was by measuring IL-8 secretion by AGS cells. These results indicated that IL-8 secretion induced by strain VM122 was ATc dependent (Figure 3A). A second means of assessing activity of the Cag T4SS was by immunoblotting to detect phosphorylated CagA in *H. pylori*-AGS co-cultures. Detection of phosphorylated CagA provides an assessment of CagA entry into cells, since only CagA that enters the host cytoplasm is phosphorylated. Phosphorylated CagA was not detected among AGS cells cultured in the absence of *H.*

pylori or among AGS cells cultured with *H. pylori* strain VM122 in the absence of ATc (Figure 3B). In contrast, phosphorylated CagA was detected among AGS cells cultured with *H. pylori* strain VM122 in the presence of ATc (Figure 3B). These results indicated that CagA translocation by strain VM122 also was ATc dependent. Together, these results demonstrated that the *tetR-tetO* system of gene regulation worked as predicted in the *H. pylori*-AGS co-culture system.

Finally, we monitored the production of Cag T4SS pili at the bacterial-host interface. Strain VM122 was grown in the absence of inducer before being added to AGS cell monolayers in the absence or presence of ATc. Analyses by scanning electron microscopy revealed no detectable pili at bacterial-host interfaces in co-cultures lacking ATc (Figure 4A). In contrast, numerous pili were detected in co-cultures grown in the presence of ATc (Figure 4B and C).

4. Conclusions

This study is the first to report on the use of a tetracycline-regulated promoter in *H. pylori*. Using this system, we demonstrated that CagU and/or CagT are not only required for functioning of the *H. pylori* Cag T4SS, but also that one or both of these proteins is required for assembly of T4SS pili at the microbial-host interface. Additional studies beyond the scope of the current study are required to differentiate between CagU- and CagT-specific effects. The assembly of the T4SS pili occurs rapidly upon induction of *cagUT* expression, with pili detected as early as 2 hours after addition of the inducer, ATc. This result is in agreement with previous reports investigating the time course of *H. pylori*-induced IL-8 induction (Sharma, Tummuru et al. 1995; Eftang, Esbensen et al. 2012). In addition to being useful in time-course studies, this method provides an alternative approach (instead of knock-out and complementation) for studying gene function, and may be applied to the study of essential genes in *H. pylori*. Furthermore, with minor modifications, the application of the method could be expanded. For example, by introducing mutations into *tetR*, the method could be modified such that regulated genes are expressed in the absence of drug and repressed by the addition of drug (i.e., Tet-on) (Urlinger, Baron et al. 2000). Alternatively, by restoring expression of *ureA* (which is required for *H. pylori* colonization of animals) in TetR-producing strains, the method could be applied to regulating *H. pylori* gene expression in animal models of disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation

ATc	anhydrotetracycline
T4SS	type IV secretion system

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Highlights

- The *tet* repressor-operator system can be used to control gene expression in *H. pylori*
- agU and/or CagT are required for assembly of the type IV secretion system pili at the *H. pylori*-epithelial interface
- The assembly of the type IV secretion system pili occurs rapidly, with pili detected as early as 2 hours after induction of the *cagUT* operon

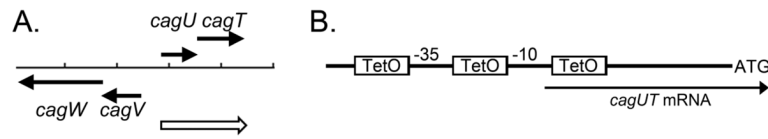


Figure 1. Introduction of *tetO* upstream of *cagUT*

A. The genetic organization of *cagUT* and flanking genes is depicted. Filled arrows represent individual open reading frames. *H. pylori* *cagU* and *cagT* are co-transcribed as part of an operon illustrated by the open arrow (Sharma et al. 2010; Ta et al. 2012). **B.** Three copies of *tetO* were introduced into the predicted promoter region upstream of *cagU*: upstream from the predicted -35 region, between the predicted -35 and -10 regions, and downstream from the identified transcription start site (Sharma et al. Nature 2010). The DNA sequence of the modified promoter region is shown in Figure S1.

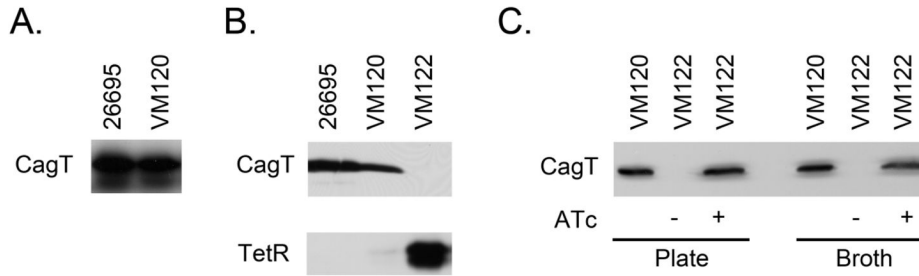


Figure 2. TetR-dependent regulation of CagT

A. Three copies of *tetO* were inserted upstream of *cagUT* in *H. pylori* strain 26695 to yield strain VM120. Samples were standardized by protein concentration and an immunoblot using anti-CagT polyclonal antiserum was used to monitor CagT production. Results indicated that the presence of the *tetO* copies upstream of *cagUT* did not disrupt CagT production. **B.** A codon-optimized copy of *tetR* was introduced into the *ureA* locus of strain VM120 to produce strain VM122. Samples were standardized by protein concentration and immunoblots using anti-CagT polyclonal antiserum or anti-TetR monoclonal antibody were used to monitor CagT and TetR production, respectively. Results indicated that CagT production was disrupted by the presence of *tetR* in strain VM122. **C.** *H. pylori* strains were cultured on agar medium or in broth culture for 16 hours in the absence or presence of ATc (100 ng ml⁻¹). Samples were standardized by protein concentration and an immunoblot using anti-CagT polyclonal antiserum was used to monitor CagT production. Results indicated that CagT production in strain VM122 could be induced by the addition of ATc.

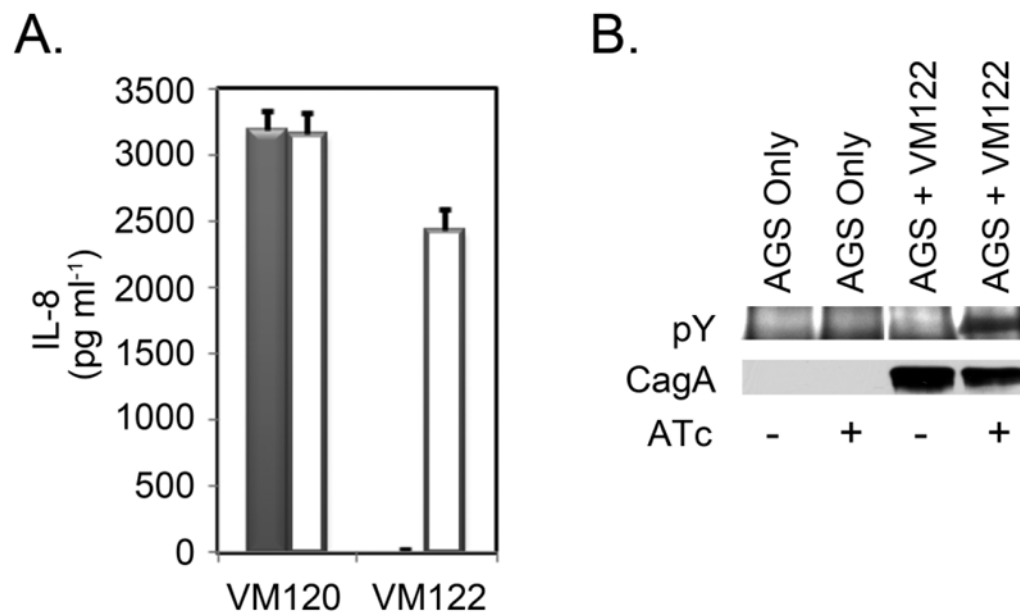


Figure 3. Inducible *H. pylori* cag type IV system function

H. pylori strains were cultured in broth for 16 hours in the absence of ATc. Bacteria then were added to AGS cell monolayers at an MOI of 100:1 in the absence or presence of ATc (200 ng ml⁻¹) and co-cultured for 7 hours. **A.** Tissue culture supernatants were collected, separated from detached cells and bacteria by centrifugation, and analysed for IL-8 content by ELISA. Results indicated that the ability of *H. pylori* strain VM122 to induce IL-8 production by AGS cells was dependent on the presence of ATc (Filled columns: co-cultures in the absence of ATc. Open columns: co-cultures in the presence of ATc) Results represent the mean and standard deviation of triplicate samples. **B.** CagA translocation was monitored by immunoblotting cell lysates using anti-phosphotyrosine (pY) antibodies and total CagA was detected using anti-CagA antibodies. Results indicated that the ability of *H. pylori* strain VM122 to translocate CagA into AGS cells was dependent on the presence of ATc.

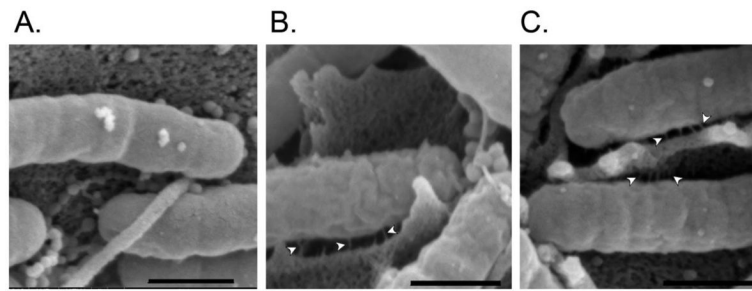


Figure 4. Inducible formation of Cag T4SS pili in an *H. pylori*-AGS cell co-culture
H. pylori strain VM122 was cultured in broth for 16 hours in the absence of ATc. Bacteria then were added to AGS cell monolayers at an MOI of 100:1 in the absence or presence of ATc (200 ng ml^{-1}) and co-cultured for 2 or 4 hours. Monolayers then were fixed and analyzed by SEM. Results indicated that the ability of *H. pylori* strain VM122 to form Cag T4SS pili at the bacterial-host interface was dependent on the presence of ATc. Representative images are shown (A: 4 hours, no ATc; B: 2 hours with ATc; C: 4 hours with ATc). Arrowheads indicate T4SS pilus structures. The bar in each image denotes 500 nm.

Table 1**Bacterial Strains and Plasmids**

Strain Designation	Description	Reference
26695	Sequenced reference strain	(Tomb, White et al. 1997)
VM120	26695 containing 3 copies of <i>tetO</i> inserted in <i>cagUT</i> promoter element, Km ^R	
VM122	VM120 containing <i>tetR</i> expressed from the <i>ureA</i> locus, Km ^R Cm ^R	
VM124	26695 containing <i>tetR</i> expressed from the <i>ureA</i> locus, Km ^R Cm ^R	
Plasmid Designation		
pAD1	Vector for introducing recombinant DNA into the <i>H. pylori ureA</i> locus	(Ando, Israel et al. 1999; Forsyth and Cover 2000; Loh, Forsyth et al. 2004; Shaffer, Gaddy et al. 2011)
pMM680	pUC57 containing the <i>H. pylori</i> 26695 <i>cagU-cagV</i> intergenic region and introduced copies of a kanamycin resistance determinant and <i>tetO</i>	
pMM682	<i>H. pylori</i> codon-optimized <i>tetR</i> cloned into pAD1	