Helicobacter pylori gene silencing in vivo demonstrates urease is essential for chronic infection

Aleksandra W. Debowski1,2,3*, Senta M. Walton1,2, Eng-Guan Chua1, Alfred Chin-Yen Tay1, Tingting Liao1,2, Binit Lamichhane1, Alfred Chin-Yen Tay1, Tingting Liao1,2, Binit Lamichhane1, Alfred Chin-Yen Tay1, Tingting Liao1,2, Binit Lamichhane1, Alfred Chin-Yen Tay1, Tingting Liao1,2, Binit Lamichhane1

1 Helicobacter pylori Research Laboratory, Marshall Centre for Infectious Disease Research and Training, School of Biomedical Sciences, University of Western Australia, Nedlands, Western Australia, Australia, 2 Ondek Pty. Ltd., Marshall Centre for Infectious Disease Research and Training, School of Biomedical Sciences, University of Western Australia, Nedlands, Western Australia, Australia, 3 School of Molecular Sciences, University of Western Australia, Crawley, Western Australia, Australia

* Current address: Swiss Vitamin Institute, Epalinges, Switzerland

* aleksandra.debowsk@uwa.edu.au

Abstract

Helicobacter pylori infection causes chronic active gastritis that after many years of infection can develop into peptic ulceration or gastric adenocarcinoma. The bacterium is highly adapted to surviving in the gastric environment and a key adaptation is the virulence factor urease. Although widely postulated, the requirement of urease expression for persistent infection has not been elucidated experimentally as conventional urease knockout mutants are incapable of colonization. To overcome this constraint, conditional H. pylori urease mutants were constructed by adapting the tetracycline inducible expression system that enabled changing the urease phenotype of the bacteria during established infection. Through tight regulation we demonstrate that urease expression is not only required for establishing initial colonization but also for maintaining chronic infection. Furthermore, successful isolation of tet-escape mutants from a late infection time point revealed the strong selective pressure on this gastric pathogen to continuously express urease in order to maintain chronic infection. In addition to mutations in the conditional gene expression system, escape mutants were found to harbor changes in other genes including the alternative RNA polymerase sigma factor, fliA, highlighting the genetic plasticity of H. pylori to adapt to a changing niche. The tet-system described here opens up opportunities to studying genes involved in the chronic stage of H. pylori infection to gain insight into bacterial mechanisms promoting immune escape and life-long infection. Furthermore, this genetic tool also allows for a new avenue of inquiry into understanding the importance of various virulence determinants in a changing biological environment when the bacterium is put under duress.
**Introduction**

The human gut pathogen *Helicobacter pylori* has coevolved with humans over thousands of years to dominate the gastric niche [1–3]. The majority of infected individuals (80–90%) carry and transmit *H. pylori* without any symptoms of disease [4, 5]. However, *H. pylori* infection causes chronic active gastritis that may develop into peptic ulceration (10–20%) or gastric adenocarcinoma (0.5–2%) [6, 7] causing a significant burden on public health [8–10]. *H. pylori* infection is persistent and clinical disease usually develops after many years of chronic inflammation and epithelial damage. Furthermore, due to increasing rates of antibiotic treatment failure [11, 12] there is a pressing need for further research into the bacterium’s mechanisms for persistence and immune evasion strategies. These are of particular importance to understanding *H. pylori* pathogenesis and to identifying novel targets for the development of new treatment options.

*H. pylori* is highly adapted to colonizing and surviving in the harsh conditions of the gastric environment. One key adaptation is the virulence factor urease. This multimeric enzyme, consisting of 12 UreA and UreB heterodimers, catalyses the hydrolysis of urea to produce CO$_2$ and NH$_3$, which acts to buffer the acidity of the local environment around the cell [13, 14]. Urease is abundantly expressed by *H. pylori* at levels exceeding that of any other known microbe [15] and is estimated to constitute 10–15% of the bacterium’s total protein content [16]. In addition, urease is essential for establishing colonization as *H. pylori* urease mutants are unable to infect the host [17–21]. Several lines of evidence suggest that urease plays a significantly greater role in infection than simple acid neutralization. Elevating the gastric pH to 7.0 was shown to be insufficient in permitting colonization by a urease negative strain [18]. In several *in vitro* studies urease and its catalytic products contributed directly to virulence. Ammonia produced by urease activity caused damage to the gastric epithelium by disrupting tight cell junction integrity [22, 23] and CO$_2$ protected against the bactericidal activity of the nitric oxide metabolite, peroxynitrite, produced by phagocytes to kill engulfed bacteria [24]. Furthermore, several studies suggest that urease may directly interact with host epithelial and immune cells. Urease has been shown to bind to major histocompatibility complex (MHC) class II molecules on gastric epithelial cells thereby inducing cell apoptosis [25] and the UreB subunit can...
stimulate monocytes to release proinflammatory cytokines by binding to cell surface CD74, a MHC class II associated invariant chain [26, 27]. In addition, an in vivo study demonstrated that changes to the surface of the urease complex resulted in the eventual clearance of H. pylori infection in mice [28]. Loss of colonization was attributed to the disruption in urea mediated interactions between H. pylori and host cells as urease activity was unaffected by the mutation, ruling out loss of acid resistance or nitrogen assimilation [29] as contributing factors [28]. Clinical isolates maintain high urease activity even after years of chronic infection when the bacterium has established itself in the relatively neutral environment of its gastric niche implicating that ongoing urease expression is required for persistence. However due to the lack of appropriate genetic systems this hypothesis could not be tested experimentally.

The necessity of urease activity in establishing colonization hinders the study of its function during persistence when using conventional knockout mutants. The availability of a conditional urease mutant would overcome this constraint by permitting changes to the urease phenotype during an established infection. To conclusively determine if urease is indeed required after colonization is established, we generated conditional H. pylori urease knockout mutants using a tetracycline repressor (tet) based system [30]. This system controls gene expression by way of a tetracycline repressor (TetR) that binds to specific operator sequences (tetO) in the target promoter and silences transcription of the downstream gene. Expression of the target gene can be turned on by the administration of a potent tetracycline inducer, such as anhydro-tetracycline (ATc) or doxycycline (Dox) [31]. This system was recently adapted to H. pylori and gene expression was regulated in vivo during active infection [32]. In the current study, we adapted this system to generate conditional urease mutants. We demonstrate that in an established infection, loss of urease expression is detrimental to the bacterial survival in the host. Strong selective pressure on the bacteria for continuous urease expression is further demonstrated by the emergence of escape mutants that successfully repopulated the mouse stomach six weeks after genetic silencing of urease was initiated.

Results

Construction and characterization of urePtetO promoters to drive urease expression

The urease structural genes, ureA and ureB, encoding for the 27 kDa UreA and 62 kDa UreB urease subunits, are transcribed as a single operon under the control of the ureA promoter, P_{ureA} [33]. To regulate the expression of urease in H. pylori, we placed the operon under tet control. Based on previous mutational studies of P_{ureA} [32, 34], the promoter was mutated to incorporate one or more tetO sequences to generate a series of different P_{ureA} derivatives, urePtetO(I-V) (Fig 1A and 1B). These tet-promoter constructs were made using PCR based techniques and used to replace the native chromosomal P_{ureA} by allelic replacement. This strategy involved first generating a recipient H. pylori strain in which P_{ureA} and ureA has been replaced with a rpsL-cat cassette. The urease negative recipient strain was then naturally transformed with the urePtetO PCR constructs to generate strains with tetO modified P_{ureA} derivatives and a restored ureA gene. H. pylori strains harbouring these constructs were characterized to identify a tet-promoter construct with regulatory properties that would permit the appropriate level of complementation to ensure colonization yet could be sufficiently silenced to prevent infection. The functionality of these tet-promoters was first assessed in the wild-type background for their ability to drive urease expression by measuring urease enzymatic activity, UreB expression and mouse colonization.

The urePtetO constructs were introduced into the wild-type X47 strain, replacing the chromosomal P_{ureA} and generating X47 urePtetO strains (OND2018—OND2022). The urease
Fig 1. Structure and functionality of tetracycline responsive ureA promoters, urePtetO. (A) Nucleotide sequence (partial) of the wild-type ureA promoter, $P_{\text{ureA}}$, and tetracycline responsive $P_{\text{ureA}}$ derivative, urePtetOIII. The -10 and -35 promoter sequences are underlined and the extended -10 region is shaded in grey. Boxes indicate tet operator (tetO) sequences. Arrow indicates the transcriptional start point (TSP). (B) Representative diagram of the urePtetO constructs. White tetO boxes indicate where the $P_{\text{ureA}}$ promoter sequence has been replaced with tetO sequences. (C) UreB expression in X47 urePtetO(I-V) strains compared to wild-type X47. Fresh cultures of $H. \text{pylori}$ grown on CBA plates were used to prepare $H. \text{pylori}$ lysates. Equal amount of protein (~15 μg) was loaded into each lane and separated on a 10% SDS–PAGE gel. Lane 1, X47 urePtetO(I), lane 2, X47 urePtetOII (II), lane 3, X47 urePtetOIII (III), lane 4, X47 urePtetOIV (IV), lane 5, X47 urePtetOV (V), lane 6, parent wild-type X47 (WT). (D) Urease activity in X47 urePtetO(I-V) strains compared to wild-type X47. Fresh cultures of $H. \text{pylori}$ grown on CBA plates for less than 24 h were collected and the urease activity for each strain was determined. Urease activity is expressed as a percentage of wild type X47 (WT) urease activity. The urePtetO construct is specified under each bar. All measurements were carried out in triplicate. Data are averages of three independent experiments and error bars represent standard deviations. (E) Two week colonization of C57BL/6J mice by X47 urePtetO(I-V) strains compared to wild-type X47 (WT). Modifications to the ureA promoter did not prevent colonization. Colonization studies were done without prior adaptation of X47 urePtetO strains to mice. Horizontal bars represent median bacterial load per group (n = 3) and points plotted represent colonization density for each individual animal. Detection limit was < 50 CFU per stomach (dotted horizontal line). Gastric specimens without $H. \text{pylori}$ re-isolation are shown as null.

https://doi.org/10.1371/journal.ppat.1006464.g001
expression level and the urease enzymatic activity of these strains under standard growing conditions was measured and compared to the parent strain (Fig 1C and 1D). Despite expressing less urease, as determined by immunodetection of UreB in the total cell lysate (Fig 1C), the urease activity measured in strains transformed with urePtetOI, urePtetOII and urePtetOV was found to be comparable to wild-type (Fig 1D). In comparison, the urease activity in strains transformed with urePtetOIII and urePtetOIV were reduced by 60% and 25% respectively, which was also accompanied by substantially reduced amount of UreB in the total cell lysate (Fig 1C).

Given that replacement of the wild type P_ureA with the urePtetO promoters resulted in reduced urease expression which concomitantly may reduce their ability to establish infection, C57BL/6j mice were challenged with strains X47 urePtetO(I-V) (OND2018—OND2022) to assess if urease expression in these strains was still sufficient to facilitate colonization. All five X47 urePtetO strains were successfully re-isolated from mouse stomachs two weeks after oral challenge (Fig 1E). The infection rate and bacterial load in mice challenged with strains X47 urePtetOI, X47 urePtetOII and X47 urePtetOV was comparable to the control group challenged with strains of lower urease activity, with strain X47 urePtetOIII displaying a major defect in colonization. To verify that colonization had not been established due to mutation or reversion of urePtetO, the ureA promoter region of re-isolated strains was sequenced which confirmed that the sequences of the urePtetO constructs remained unaltered after passage through mice.

**Tet-regulation of urease expression in H. pylori**

After establishing that the tetO modifications to the ureA promoter did not abrogate colonization per se, we evaluated if these promoters could regulate urease expression in a tetracycline dependent manner. All five ureA promoter derivatives were transformed into a X47 recipient strain that expressed TetR under the control of the strong flaA promoter [32]. The resulting strains, X47 mdaB::ptetR4, urePtetO(I-V) (OND1954—OND1958), did not express urease when grown on standard CBA plates however urease expression could be induced when grown on CBA plates containing 50 ng/ml anhydrotetracycline (ATc) (S1 Fig). These results demonstrated that TetR effectively silenced the tet-promoters in these strains.

**Characterization of urePtetO regulated urease expression and activity in vitro**

Regulation of urePtetO promoters by TetR in conditional urease knockout strains X47 mdaB::ptetR4, urePtetO(I-V) was assessed using the urease enzymatic activity assay. Bacteria were cultured in the absence or presence of 50 ng/ml ATc for two successive passages and then collected for analysis. When strains were cultured in the absence of ATc, urease activity was below the detection limits of the assay (2 U/ml of Type III urease from Jack bean) (Fig 2A). For strains grown in the presence of ATc, urease activity in strains X47 ptetR4; urePtetO-I, -II and -V was induced to wild type levels, while the urease activity for strains X47 ptetR4; urePtetO-III and -IV remained below 10% of wild-type activity. These results demonstrated that by using the appropriate tet-promoter urease activity can indeed be regulated by the presence of a small molecule inducer, confirming the generation of conditional H. pylori urease knockout mutants.

The tet-responsive promoters urePtetOI and urePtetOV have different genetic architectures (Fig 1B) and upon induction also promoted the greatest urease expression levels amongst the tested strains. Based on these results the regulation of these two promoters was further characterized. The kinetics of urePtetO induction and repression was analysed in strain X47 ptetR4;
urePtetOI (OND1954) and X47 ptetR4; urePtetOV (OND1958) by immunodetection of the UreB protein (Fig 2B and 2C). After addition of 200 ng/ml ATc to the culture medium, UreB protein expression increased over time and reached maximum levels after 12 h and 8 h for urePtetOI and urePtetOV, respectively (Fig 2B). Withdrawal of ATc from induced cultures led to a significant decrease in UreB protein levels within 3 h, demonstrating that both urePtetOI and urePtetOV were quickly silenced (Fig 2C) and that the UreB protein was turned over efficiently, falling to the threshold of detection within 12 h.

Establishing a conditional urease knockout infection model

With the knowledge that tetracycline dependent regulation of urease expression was attainable in vitro we next turned our attention to establishing a mouse model of infection. Based on previous studies involving in vivo tet-systems [35–37] the inducer molecule doxycycline (Dox) was first used as a model inducer to identify a maximal dosage of material that could be tolerated by the bacterium in vivo. We found that wild-type X47 could still infect mice when the animals were supplemented with up to 10 mg/l of Dox in their drinking water. Colonization by wild-type X47 was severely attenuated at 100 mg/l of Dox and bacteria could not be reisolated at 1000 mg/l of Dox supplement (S2A Fig). Furthermore, strain X47 ptetR4; urePtetOI (OND1954), which emerged as the prime conditional urease mutant candidate from the in vitro studies, was tested to verify its ability to establish initial colonization and then used to optimise the dosage of inducer molecule to regulate urease expression in vivo. OND1954 was only capable of establishing infection in C57BL/6J mice when the animals received Dox supplementation in their drinking water, demonstrating that urease is essential for OND1954 to establish colonization in the mouse infection model (S2B Fig). Addition of Dox supplement at 1 mg/l supported colonization of OND1954 and although attenuated, the conditional mutant was also isolated from animals supplemented with Dox at 5 mg/l and 10 mg/l.
Having identified a minimal supplement dose of Dox inducer, we then sought to complete the infection model by investigating two more important factors; the use of the less toxic tetracycline derivative ATc, and attempting to improve the robustness of the conditional urease mutant strain. The original wild-type X47 strain underwent four consecutive transformations to generate the conditional urease mutant OND1954 and consequently the strain may have accumulated secondary mutations that would decrease its fitness in vivo. To address this, the output clones of OND1954 isolated from three individual mice were collected and each clone was verified to be a conditional urease mutant. These clones were then pooled, OND3241 (A-E), and used in subsequent infection studies to test if passage through mice led to improved infection rates. Mice were challenged with either the wild-type strain, the original conditional mutant OND1954 or the mouse passaged urease conditional mutant OND3241, and supplemented without or with 5 mg/l Dox or ATc. Colonization of OND3241 remained dependent on inducer supplement and using ATc instead of Dox resulted in an improved infection rate and bacterial load in the infection model (Fig 3).

Fig 3. Infection of conditional urease mutant is dependent on tetracycline inducers. Optimization of in vivo model for tet-regulated urease expression. Mice groups were supplemented without (C) or with 5 mg/l of either Dox or ATc in their drinking water. Animals were orally challenged with wild-type strain X47, with pre-induced OND1954 or a pool of pre-induced clones of OND1954 re-isolated from mice (OND3241A-E). Animals were sacrificed two weeks after oral challenge. Bars represent median bacterial load per group (n = 5) and points plotted represent colonization density for each individual animal. Detection limit was < 50 CFU per stomach (dotted horizontal line). Gastric specimens without H. pylori re-isolation are shown as null. Statistical analysis was performed to test if Dox or ATc treatment influenced the infection rate of any of the given bacterial strains. Treatment did not significantly impact on the infection rate of the wild-type strain. However ATc supplementation significantly increased the infection rate for OND1954 and OND3241 (* p < 0.05). Unpaired two sided Fisher’s exact test followed by Bonferroni correction was used for pairwise testing of the infection status.

https://doi.org/10.1371/journal.ppat.1006464.g003
Silencing urease during active infection

Having established an infection model in which the \textit{H. pylori} urease phenotype could be regulated \textit{in vivo}, we proceeded to investigate what effect \textit{tet}-mediated silencing of urease expression had on established \textit{H. pylori} infections. Mice were challenged with the conditional strain OND3241 and provided with 5 mg/l ATc supplement to establish infection. After two weeks, the supplement was withdrawn and the animals were sacrificed at indicated time points. The conditional \textit{H. pylori} urease mutant could still be isolated on days 1 and 3 after supplement withdrawal, however on days 5 and 7 the bacterial load had decreased to below our detection limit (Fig 4A). This data demonstrated for the first time that continuous urease expression is required by \textit{H. pylori} to maintain colonization even after the bacteria have become established in the gastric niche.

Isolation of escape mutants demonstrates strong selective pressure for urease expression

To test if \textit{H. pylori} were under selective pressure to overcome \textit{tet}-regulation the suppression experiment was repeated and the animals were sacrificed at a much later time point. Mice were challenged with either wild-type X47 or OND3241 and provided with 5 mg/l ATc supplement to establish infection. After two weeks, the supplement was withdrawn from half the groups (both OND3241 and wild-type) while the remaining groups were maintained on ATc supplement and the animals were sacrificed at different time points (Fig 4).

No differences in bacterial load or infection rate was observed for animals infected with wild-type X47 over the course of the experiment demonstrating that long-term ATc supplement (5 mg/l for 8 wks) does not interfere with \textit{H. pylori} infection (Fig 4B). Animals challenged with OND3241 and maintained continuously on ATc supplement had a consistent infection rate of 60% (Fig 4B). \textit{In vitro} tests confirmed that bacteria re-isolated from these groups remained conditional urease mutants, even after a total infection time of 8 weeks. Withdrawal of ATc supplement from the animal groups challenged with OND3241 resulted in reduced infection load on days 3 and 5 and, although not completely cleared to below our detection limit in all animals, the infection rate had decreased to 20% on day 5. However, when mice challenged with the OND3241 were left in the absence of ATc for 42 days, the bacterial load and the infection rate had increased resulting in 80% of the animals bearing bacteria in the stomach above our detection limit. Importantly, unlike the bacteria re-isolated at the earlier time points (day 3 and day 5), \textit{H. pylori} re-isolated from this last group of mice were all urease positive and they were no longer conditional urease mutants as tested qualitatively \textit{in vitro}. This result revealed that the strain was under selective pressure to restore urease expression.

In an effort to identify possible genetic mutations to overcome \textit{tet}-regulation of the urease operon, whole genome sequencing of the original conditional urease mutant strain OND1954, the individual clones of input strain OND3241(A-E), and 34 output strains recovered at day 42 (5 conditional urease-negative strains and 29 urease-positive \textit{tet}-escape mutants) was undertaken. Sequence data were mapped against reference strain OND1954 and variants specific to \textit{tet}-escape mutants were identified (S1 Table). Sequence analysis of the \textit{ureAB} locus, including the upstream regulatory region, revealed no changes between OND1954, OND3241 and the \textit{tet}-escape mutants. Interestingly, the \textit{tet}-escape mutants harboured non-synonymous substitutions or frameshift mutations in at least one of the following genes, \textit{tetR} and the flagellar biosynthesis genes \textit{fliA}, \textit{fliE} and \textit{flgE}. These data reveal that \textit{tet}-regulation was overcome in the \textit{tet}-escape mutants not by altering the \textit{tetO} binding sites but through affecting the repressor protein.
Fig 4. Tet-regulated silencing of urease expression during active infection. Urease expression in conditional urease mutant strains OND3241 was induced with 50 ng/ml ATc for 48 h prior to oral challenge. (A) Mice were orally challenged with pre-induced OND3241 and supplemented with 5 mg/l ATc for two weeks. ATc supplement was maintained (+, ■) or withdrawn (+/-, □) and animals were sacrificed 0, 1, 3, 5 and 7 days later. Bars represent median bacterial load per group (n = 5) and points plotted represent colonization density for each individual animal. Detection limit was < 50 CFU per stomach (dotted horizontal line). Gastric specimens without H. pylori re-isolation are shown as null. (B) Mice challenged with wild-type X47 (WT, ●) or pre-induced OND3241 were supplemented with 5 mg/l ATc for two weeks. After this two week period, ATc supplement was maintained (+, ■) or withdrawn (+/-, □) and animals were sacrificed 3, 5 and 42 days later. Bars
Discussion

A set of conditional urease mutants were generated to demonstrate for the first time that urease expression is essential in the persistence stage of *H. pylori* infection, which broadens our understanding of the role of this enzyme during chronic infection.

Genetic manipulation of *P.ureA* to place urease under tet-control led to a decrease in the basal levels of urease expression for all *urePtetO* constructs tested. However, under the conditions tested, strains transformed with *urePtetOI*, *urePtetOII* and *urePtetOV* were found to have comparable urease activity to that of wild-type. This data can be reconciled as it has been reported that under neutral *in vitro* growth conditions without added nickel, such as the growth conditions used in this study, a significant amount of urease in wild-type strains is in the inactive apoenzyme form and only a minor fraction of urease is active [38–40]. Urease activity in *H. pylori* is highly controlled and is modulated through several different mechanisms in response to various environmental cues [39]. Since our goal was to establish a working *in vivo* model, we decided to directly test if the decrease in urease expression could be tolerated by the bacteria by assessing if the *urePtetO* strains were capable of colonizing the murine stomach.

Interestingly, when analyzing the *in vivo* colonization data from the X47 *urePtetO* strains (OND2018—OND2022) a positive correlation between infection rate and *in vitro* urease expression and activity but not to bacterial load in colonized animals was observed (Fig 1E). This finding suggests that for initial colonization of the murine stomach the amount of urease activity is an important factor likely due to the fact that the bacteria need to withstand the acidity of the gastric lumen until they reach their gastric niche, deep into the gastric mucus near the epithelial surface. However, once the bacteria are established within their environmental niche, although urease is still required for growth, the level of urease expression may be less important for maintaining colonization as mice colonized with strains transformed with *urePtetOIII* and *urePtetOIV* had a similar bacterial burden compared to mice infected with strains expressing more urease.

Infection of the mouse host by the conditional urease mutant was strictly dependant on supplementation with a tetracycline inducer, confirming that genetic regulation of urease expression was stringent enough to prevent colonization. Furthermore, in the induced state, tet-mediated expression of urease was sufficient to allow and maintain infection by the conditional urease mutant. Withdrawal of the supplement resulted in clearance of the bacterium within 5 days. This is in line with the slow shut-off observed in other mouse models using tet-based regulation systems which has been attributed to the persistence of doxycycline in tissues [41, 42].

Notably the longer time of clearance of the bacterium *in vivo* provided the opportunity for the emergence and selection of tet-escape mutants. *H. pylori* possess several mechanisms, such as an error prone PolA [43] and efficient DNA homologous recombination and transformation systems [44, 45], that permit the bacterium to undergo rapid microevolution to adapt to changing environments in its specific host [46, 47]. The emergence of tet-escape mutants in this study suggests that there is strong selective pressure on the bacterium for continuous
urease expression to maintain chronic infection. Whole genome sequence analysis of the tet-escape mutants identified several different mutations that likely explain how tet-regulation of the ureAB operon was overcome.

One group of escape mutants had missense or nonsense mutation within the tetR gene. TetR is a finely tuned transcriptional regulator [31] and therefore most amino acid changes are likely to have deleterious effects to the function of TetR by inhibiting repressor dimerization or DNA binding [48–50]. Interestingly, another group of tet-escape mutants harbored either amino acid substitutions within the DNA binding domains of the alternative RNA polymerase sigma factor FliA (g^{28}) or a truncated FliA due to nonsense mutation and frameshift alteration. FliA controls the transcription of some late flagellar genes (class 3) including the flagellin subunit, flaA [51, 52]. Previous studies have reported that H. pylori flaA mutants have no detectable flaA transcript and have truncated flagella [51]. In the conditional H. pylori urease mutants generated in this study, the transcription of tetR is driven by a flaA promoter. Therefore, it is reasonable to suggest that the flaA mutations identified in the tet-escape mutants likely affect the transcription of tetR from P_{flaA} and consequently release the ureAB operon from tet-regulation. Additionally, some tet-escape mutants acquired non-synonymous mutations in fliE and flgE, genes that encode for components of the flagellum hook-basal body complex. Flagellar biosynthesis is a highly ordered and regulated process and transcription of late flagellar genes by flaA proceeds only once the hook-basal body complex is complete [53, 54]. Mutation of flgE has been shown in other studies to affect the regulation of flaA dependent genes, leading to a two-fold reduction in flaA transcript levels [54]. Thus mutation of fliE and flgE may indirectly impact on the transcription of tetR from P_{flaA} through the negative control of FliA [51, 53].

Motility is essential for H. pylori colonization [19, 55, 56], and therefore the isolation of tet-escape mutants with mutations in genes involved in flagellar biosynthesis (fliA, fliE and flgE) raises interesting questions. During the chronic stage of infection, once the bacteria are established in their environmental niche and have adhered to gastric epithelial cells [57], can motility be compromised in preference for improved urease expression? Further investigations with the appropriate conditional mutants are necessary to better understand the escape and in particular to test whether motility is still required once colonization is established. Another question is whether there are other mutations acquired by the tet-escape mutants that could compensate for mutations in fliA, fliE and flgE? In-depth analysis of the whole genome sequencing data together with mutational studies needs to be undertaken to discern how these tet-escape mutants are able to survive in the host.

The sequencing results underline the importance of urease for H. pylori to maintain persistence infection and reveal the high selective pressure for continuous expression of urease even after colonisation is successfully established. The mutations identified in the tet-escape mutants add support to the hypothesis that the genomic plasticity of H. pylori is an important mechanism for adaptation to new and changing environments [58]. Furthermore these findings also highlight the potential of using tet-based genetic tools together with whole genome sequencing to study H. pylori genetic plasticity and adaptation in a changing biological environment when the bacterium is put under duress.

Using the conditional mutant, we have demonstrated that urease is essential for chronic infection and that repression of urease expression results in the loss of bacterial load within 5 to 7 days. The availability of these tools now allows for new questions to be asked regarding the reason behind the relatively rapid loss in colonization. One reason may be that the loss of urease activity results in the bacteria being more susceptible to clearance by phagocytic cells [24]. Another possibility is that the loss of urease activity negatively affects H. pylori ability to swim through gastric mucus, as the bacteria would no longer have the ability to decrease the viscosity of mucus through the elevation of local pH [59], and consequently are cleared due to turnover.
of the mucus lining. The conditional urease mutant will serve as a valuable tool in further studies that pursue this line of investigation.

In this study, *H. pylori* conditional urease mutants were generated by placing the expression of the urease subunits, UreA and UreB, under *tet*-control and have permitted the first direct testing of the hypothesis that urease is required by *H. pylori* for chronic infection. Furthermore, eventual escape from *tet*-regulated urease expression by *H. pylori* demonstrates that there is a very strong selective pressure on the bacterium to maintain urease expression during infection. Our data validates urease as a good target for therapeutic intervention. The conditional urease mutants generated here can also be used to gain more detailed insight into the role of urease in the persistence stage of infection including its interactions with MHC class II molecules [25], induction of proinflammatory cytokine [27] and its potential role in motility [59]. Furthermore, this study demonstrates the need for conditional mutants, generated by using genetic tools such as the *tet*-system, to study *H. pylori* virulence factors, persistence and the bacterium’s influence on the host microbiota.

**Materials and methods**

**Bacterial strains and culture conditions**

*H. pylori* X47 strains used in this study are listed in S2 Table. Bacteria were grown at 37°C under microaerobic conditions on Columbia blood agar (CBA) plates containing 5% horse blood and Dent’s antibiotic supplement (Oxoid). When appropriate, antibiotic selection was carried out by supplementing media with chloramphenicol or streptomycin at a final concentration of 10 μg/ml. Microaerobic conditions were established in sealed jars using the Anoxomat MarkII system (Mart Microbiology B.V., the Netherlands) after one atmosphere replacement with the following gas composition N₂:H₂:CO₂, 85:5:10.

**Genetic manipulation and *H. pylori* strain construction**

All genetic manipulation of *H. pylori* strains was done using genomic insertion and replacement of a counter-selectable *rpsL-cat* cassette [60]. The use of the counterselectable streptomycin susceptibility (*rpsL*-based) system requires a host strain that possesses a streptomycin-resistant phenotype [61]. The *H. pylori* X47 host strain is naturally streptomycin-resistant and no modifications to this strain were required. The genotype of all mutants was confirmed by PCR and/or DNA sequencing.

**Oligonucleotides**

Oligonucleotides used in this study are listed in S3 Table.

**Construction of the *H. pylori* tetracycline responsive *urePtetO* promoters**

To place *ureA* and *ureB* under *tet* control, wild-type nucleotide sequences flanking the -35 and -10 promoter regions of the *ureA* promoter, P*ureA*, were replaced with *tetO* sequences to generate five derivatives of P*ureA*, *urePtetO*(-I through -V) (Fig 1A and 1B). These promoter constructs were used to replace the native urease promoter, using the two-step *rpsL-cat* based transformation approach.

**Construction of *ureA::rpsL-cat* recipient strain**

A construct composed of the counterselection cassette flanked by DNAs homologous to regions of the *ureA* locus, *ureA::rpsL-cat*, was made by SOE PCR [62, 63] (S3 Fig) and used to generate recipient strains in which P*ureA* and *ureA* were replaced with *rpsL-cat*. Two 1 kb
regions flanking P\textsubscript{ureA} and ureA (HP0073), were amplified from 26695 genomic DNA using primers ureArcat1 and ureArcat2, and ureArcat3 and ureArcat4 respectively. The \textit{rpsL-cat} selection cassette was amplified using primers ureArcat5 and ureArcat6. Nested primers ureArcat7 and ureArcat8, were used to generate and amplify a final 3.4 kb PCR product, \textit{ureA::rpsL-cat}. Natural transformation of the \textit{H. pylori} strains with the \textit{ureA::rpsL-cat} PCR construct was performed to obtain the recipient strain OND2017. Transformants isolated on chloramphenicol plates were urease negative.

Reconstruction of \textit{ureA} promoter to incorporate \textit{tetO} sites and generation of conditional urease mutants

Five \textit{tetO} modified \textit{ureA} promoter constructs \textit{urePtetO(I-V)}, containing up to three \textit{tetO} sites, were constructed by SOE PCR (S4 Fig). The primer pairs used to make each \textit{urePtetO} construct are listed in S4 Table. Briefly, a 1 kb fragment upstream, arm I, and a 1.5 kb fragment downstream, arm II, of P\textsubscript{ureA} were amplified using 26695 genomic DNA as a template. Long primer tails were used to reconstruct the \textit{ureA} promoter region upon fusion of arms I and II by SOE PCR. Primers ureArcat7 and ureArcat8 were used to amplify the final 2.5 kb products, \textit{urePtetO(I-V)}, and sequencing confirmed that the modified \textit{ureA} promoters were reconstructed correctly. Natural transformation of the recipient strain OND2017 with \textit{urePtetO} PCR constructs resulted in replacement of the \textit{rpsL-cat} with \textit{urePtetO} and restoration of \textit{ureA}, generating strains X47 \textit{urePtetO} through X47 \textit{urePtetO}V (OND2018—OND2022). Correct allelic replacement of the resulting \textit{Str\textsuperscript{r}} transformants was confirmed by colony PCR using primers ureAP1 and ureArcat8 and by sequencing using primer urePseq.

Conditional urease strains were generated by transforming the TetR expressing \textit{H. pylori} strain, X47 \textit{mdaB::ptetR}4 (OND1987) [32], with the \textit{ureA::rpsL-cat} PCR construct to generate the recipient strain OND2026. This urease negative strain was then transformed with each of the five \textit{urePtetO} constructs to generate conditional urease mutant strains X47 \textit{mdaB::ptetR}4; \textit{urePtetO(I-V)} (OND1954—OND1958). Transformants were first screened for tetracycline dependent urease expression using the urease phenotype assay and additional characterization was done using the urease activity assay and immunoblot analysis.

Urease phenotype assay

Urea culture plates (Brucella broth, 7% NCS, 1 mM urea, phenol red 100 mg/l, vancomycin 6 mg/l, pH 6) were used to assay the urease phenotype of \textit{H. pylori} clones. The pH of the media was adjusted with 1 M HCl before the addition of NCS and vancomycin. The pH was low enough to observe the colourimetric change of phenol red, from yellow to red, due to the catalytic activity of urease on urea, but not acidic enough to inhibit the growth of urease negative strains. To screen for \textit{tet}-regulated urease activity, transformants and colonies re-isolated from infected animals were replica plated onto CBA plates with or without 50 ng/ml of ATc and cultured for 48 h. Bacteria were then patched onto urea plates and incubated under microaerobic conditions. Urea plates were examined after 16 h of incubation to identify clones that had switched urease phenotype upon exposure to ATc. Localized changes in colour around each growing colony identified urease positive clones. Conditional urease mutant strains grown on CBA plates without ATc remained urease negative, while strains grown on CBA plates with ATc became urease positive (Example S5 Fig).

Measurement of urease enzymatic activity

The urease activity assay used in this study was adapted from the protocol previously described [28]. Strains were grown on CBA plates without or with 50 ng/ml ATc for two successive
passages. Bacteria from 24 h plate cultures were collected and resuspended in cold buffer A (25 mM phosphate buffer, pH 6.8) and standardized to an OD$_{600}$ = 4.0. A 50 μl aliquot of the standardized bacterial suspension was then diluted with 50 μl of buffer B (25 mM phosphate buffer, pH 6.8, 0.2% Tween-20). A 25 μl aliquot of this diluted bacterial suspension was transferred into one well of a 96 well plate, diluted with 150 μl of buffer C (25 mM phosphate buffer, pH 6.8, 250 μM phenol red) and incubated for 5 min at 37˚C. A 75 μl aliquot of urea solution (0.5 M) was then added to the well and the absorbance at 560 nm was measured every 72 s for 75 cycles using a POLARstar Omega (BGM Labtech) plate reader. Activity was measured as the rate of change in absorbance over time and expressed as percent of urease activity of the wild-type X47 strain. All urease activity measurements were carried out in triplicate and experiments were repeated at least three times.

**Time course experiments of tet-regulation**

Bacteria were grown in Heart Infusion (HI) medium supplemented with 10% Newborn Calf Serum (NCS) and vancomycin (6 μg/ml). Cultures were inoculated with bacteria suspended in PBS to give a starting OD$_{600}$ = 0.05, and grown under microaerobic conditions at 37˚C and 120 rpm. For induction, *H. pylori* cultures were grown to mid-log phase in 10 ml of media. Cultures were induced with 200 ng/ml ATc and bacteria were incubated for another 12 h, with aliquots were taken at indicated time points. For gene silencing, conditional strains were cultured in the presence of 200 ng/ml ATc to mid-log phase. Fresh HI media, with or without 200 ng/ml ATc, was inoculated with pre-induced bacteria (OD$_{600}$ = 0.5) and grown for 12 h, with aliquots taken at indicated time points. Bacterial cells were collected by centrifugation and washed twice with PBS before processing for immunoblot analysis.

**SDS-PAGE and immunoblot analysis**

Bacterial whole cell lysates were prepared as previously described [32]. The protein concentration of bacterial cell whole cell lysate samples was determined using the Micro BCA protein assay reagent kit (Pierce) with bovine serum albumin as the standard. Equal amounts of protein for each sample were mixed with 3x SDS-PAGE sample loading buffer, incubated at 95˚C for 10 min, and proteins were separated by 10% SDS-PAGE and electrotransferred to a PVDF membrane. For detection of the UreB subunit of urease, mouse anti-UreB (Austral biologicals) was used at a dilution of 1:8000. Secondary antibody rabbit anti-mouse-HRP (Jackson ImmunoResearch Laboratories) was used at a dilution of 1:10,000 and detection of the secondary HRP conjugate was accomplished by chemiluminescence (Sigma) using LAS 3000 (Fujifilm) (software Image reader LAS 3000 V2.2). For loading controls, duplicate gels were run in parallel and stained with Coomaise Brilliant Blue R-250 (S1B Fig, S6 Fig and S7 Fig).

**Animal experiments**

Mouse procedures were reviewed and approved by the Institutional Animal Care and the Animal Ethics Committee of the University of Western Australia. 6–7 week old C57BL/6j female mice were challenged once by oral gavage with 200 μl of 1 x 10$^9$ CFU/ml of bacteria suspended in HI broth. Groups of infected mice received doxycycline (Dox), anhydrotetracycline (ATc) or no supplement in drinking water containing 5% sucrose. Water was kept in light-protected bottles and changed every three days. Mice were sacrificed at indicated time points and stomachs were removed and homogenized in 1 ml HI using a tissue lyser (Retch). Homogenates were serially diluted and plated out on *H. pylori* selective plates (CBA containing 5% Horse blood, Dent, nalidixic acid 10 mg/l and Bacitracin 100 mg/l) to determine the bacterial burden. Were appropriate, re-isolated clones were assayed for tet-responsive gene expression.
Illumina library preparation and sequencing

Preparation of MiSeq library was performed using Illumina Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA) as previously described with minor modifications [64]. In brief, 1 ng of genomic DNA was fragmented in 5 μl of Amplicon Tagment Mix and 10 μl of Tagment DNA buffer. Tagmentation reaction was performed by incubation at 55˚C for 10 min followed by neutralisation with 5 μl of Neutralise Tagment Buffer for 5 min. Tagmented DNA (25 μl) was indexed in a 50 μl limited-cycle PCR (12 cycles) as outlined in the Nextera XT protocol and subsequently purified using 25 μl of AMPure XP beads (Beckman Coulter Inc, Australia). The fragment size distribution of the purified DNA was analysed utilising a LabChip GXII 2100 Bioanalyser. DNA libraries were adjusted to 2 nM, pooled in equal volumes and then denatured with 0.2 N NaOH according to the Nextera protocol. The libraries were sequenced using 2 × 300 paired-end protocols on an Illumina MiSeq instrument (MiSeq Reagent Kit v3 for 600 cycles). The draft genome sequence of *H. pylori* OND1954 has been deposited at DDBJ/ENA/GenBank under the accession MVFB00000000. The version described in this paper is version MVFB01000000. All raw sequence data generated in this study have been submitted to Sequence Read Archives (SRA) database with accession numbers listed in S5 Table.

Sequence data de novo assembly, annotation, reference mapping and variant calling

The generated MiSeq reads of *H. pylori* strain OND1954 was assembled using SPAdes genome assembler (version 3.8.2) with careful option [65]. The draft genome sequence was subsequently annotated using Prokka (version 1.11) with Swiss-Prot, Pfam (release 30.0), TIGRFAMs (release 15.0) and Superfamily (version 1.75) databases [66–70]. The annotation features are available in S1 File. The raw reads of OND1954-derivative strains were trimmed and mapped against the annotated draft genome using Bowtie2 on Geneious R7 platform [71, 72]. Variants were called using the following parameters: minimum coverage = 10 and minimum variant frequency = 0.7.

Statistical analysis

For mouse colonization assays (where n ≥ 5) the Mann-Whitney unpaired two-tailed test was used to compare colonization loads and the two sided Fisher’s exact test was used to compare infection rates. Bonferroni correction was used for multiple pairwise testing. Statistical analysis was performed using GraphPad Prism version 7 for Windows, (GraphPad Software) and Stata Statistical Software (StataCorp. 2015. *Release 14*. College Station, TX: StataCorp LP.).

Supporting information

**S1 Fig. TetR silencing of urePtetO in *H. pylori***. UreB protein was detected in *H. pylori* strains harbouring *urePtetO* and expressing TetR under the control P_{flaA} (OND1954—OND1958). Bacteria were cultured on standard CBA plates or CBA plates containing 50 ng/ml ATc for 48 h and fresh bacteria cultures were used to prepare whole cell lysates. Equal amount of protein was loaded into each lane and separated on a 10% SDS–PAGE gel. (A) The *urePtetO* construct is specified under the bars. UreB protein could not be detected in samples from bacteria grown in the absence of ATc. UreB expression was strongly induced in strains harbouring *urePtetOI*, *urePtetOII* and *urePtetOIV*. Induction of UreB expression was weaker in strains harbouring *urePtetOIII* and *urePtetOIV*. (B) Coomassie stain of duplicate gel. (TIF)
**S2 Fig. H. pylori X47 tolerance to Dox in vivo.** (A). Mice were orally challenged with wild-type X47 strain and supplemented with a range of Dox concentrations (1, 10, 100 and 1000 mg/l) in their drinking water. Bars represent median bacterial load per group and points plotted represent colonization density for each individual animal. Detection limit was < 50 CFU per stomach (dotted horizontal line). Gastric specimens without *H. pylori* re-isolation are shown as null. (B) Urease expression in conditional urease mutant strain OND1954 was induced with 50 ng/ml ATc for 48 h prior to oral challenge. Mice were orally challenged with wild-type X47 strain or pre-induced OND1954 and supplemented with a range of Dox concentrations (1–20 mg/l) in their drinking water. Animals were sacrificed one week after oral challenge. Bars represent median bacterial load per group (n = 3) and points plotted represent colonization density for each individual animal. Detection limit was < 50 CFU per stomach (dotted horizontal line). Gastric specimens without *H. pylori* re-isolation are shown as null.

(TIF)

**S3 Fig. Diagram of PCR construction strategy for ure::rpsL-cat.**

(TIF)

**S4 Fig. Diagram of PCR construction strategy for urePtetO.**

(TIF)

**S5 Fig. Assay for urease activity using acidified urea plates.** To screen for *tet*-regulated urease activity, *H. pylori* clones were replica plated onto CBA plates without (control) or with 50 ng/ml of ATc and cultured for 48 h. Bacteria were then patched onto urea plates and incubated under microaerobic conditions. An example from testing clones of strain OND1954 is provided. Change in colour due to urease activity is shown after 0.25 h, 2 h and 16 h of incubation. Conditional urease mutant strains grown on CBA plates without ATc remained urease negative, while strains grown on CBA plates with ATc became positive for urease activity.

(TIF)

**S6 Fig. Loading control for Fig 1C.** Coomassie stain of duplicate gel for UreB expression in X47 urePtetO(I-V) strains compared to wild-type X47.

(TIF)

**S7 Fig. Loading controls for Fig 2B and 2C time course experiments.** Coomassie stain of duplicate gels for time course of TetR-controlled expression of UreB in conditional urease mutants OND1954 and OND1958.

(TIF)

**S1 Table. CDS mutations identified in *tet*-escape mutants.**

(XLSX)

**S2 Table. Strains used in this study.**

(DOCX)

**S3 Table. Oligonucleotide primers used in this study.**

(DOCX)

**S4 Table. Oligonucleotide primer pairs used to generate urePtetO constructs.**

(DOCX)

**S5 Table. Strain SRA accession no.**

(DOCX)
S1 File. OND1954 genome sequence with annotated features.
(GFF)

Acknowledgments
Michelle Middleton and Tania Camilleri are thanked for technical support.

Author Contributions
Conceptualization: Aleksandra W. Debowski, Barry J. Marshall, Mohammed Benghezal.
Data curation: Aleksandra W. Debowski, Senta M. Walton, Eng-Guan Chua, Alfred Chin-Yen Tay.
Formal analysis: Aleksandra W. Debowski, Senta M. Walton, Eng-Guan Chua, Alfred Chin-Yen Tay, Binit Lamichhane.
Funding acquisition: Aleksandra W. Debowski, Senta M. Walton, Keith A. Stubbs, Barry J. Marshall.
Investigation: Aleksandra W. Debowski, Senta M. Walton, Tingting Liao, Binit Lamichhane, Robyn Himbeck.
Methodology: Aleksandra W. Debowski, Senta M. Walton, Robyn Himbeck.
Project administration: Aleksandra W. Debowski, Senta M. Walton, Alfred Chin-Yen Tay, Alma Fulurija, Mohammed Benghezal.
Resources: Alfred Chin-Yen Tay, Keith A. Stubbs.
Supervision: Keith A. Stubbs, Mohammed Benghezal.
Validation: Aleksandra W. Debowski, Senta M. Walton, Keith A. Stubbs, Mohammed Benghezal.
Visualization: Aleksandra W. Debowski, Senta M. Walton, Keith A. Stubbs, Mohammed Benghezal.
Writing – original draft: Aleksandra W. Debowski, Mohammed Benghezal.
Writing – review & editing: Aleksandra W. Debowski, Senta M. Walton, Eng-Guan Chua, Keith A. Stubbs, Mohammed Benghezal.

References
1. Andersson AF, Lindberg M, Jakobsson H, Backhed F, Nyren P, Engstrand L. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS One. 2008; 3(7):e2836. https://doi.org/10.1371/journal.pone.0002836 PMID: 18665274
2. Bik EM, Eckburg PB, Gill SR, Nelson KE, Purdom EA, Francois F, et al. Molecular analysis of the bacterial microbiota in the human stomach. Proc Natl Acad Sci U S A. 2006; 103(3):732–7. https://doi.org/10.1073/pnas.0506651103 PMID: 16407106
3. Linz B, Balloux F, Moodley Y, Manica A, Liu H, Roumagnac P, et al. An African origin for the intimate association between humans and Helicobacter pylori. Nature. 2007; 445(7130):915–8. https://doi.org/10.1038/nature05562 PMID: 17287725
4. Blaser MJ, Parsonnet J. Parasitism by the "slow" bacterium Helicobacter pylori leads to altered gastric homeostasis and neoplasia. J Clin Invest. 1994; 94(1):4–8. https://doi.org/10.1172/JCI117336 PMID: 8040281
5. Morris AJ, Ali MR, Nicholson GI, Perez-Perez GI, Blaser MJ. Long-term follow-up of voluntary ingestion of Helicobacter pylori. Ann Intern Med. 1991; 114(8):662–3. PMID: 2003713
6. Atherton JC. The pathogenesis of Helicobacter pylori-induced gastro-duodenal diseases. Annu Rev Pathol. 2006; 1:63–96. https://doi.org/10.1146/annurev.pathol.1.110304.100125 PMID: 18039108

7. Kusters JG, van Vliet AH, Kuipers EJ. Pathogenesis of Helicobacter pylori infection. Clin Microbiol Rev. 2006; 19(3):449–90. https://doi.org/10.1128/CMR.00054-05 PMID: 16847081

8. Herrero R, Park JY, Forman D. The fight against gastric cancer—the IARC Working Group report. Best Pract Res Clin Gastroenterol. 2014; 28(6):1107–14. https://doi.org/10.1016/j.bjp.2014.10.003 PMID: 25439075

9. Oh JK, Weiderpass E. Infection and cancer: global distribution and burden of diseases. Ann Glob Health. 2014; 80(5):384–92. https://doi.org/10.1016/j.ajog.2014.09.013 PMID: 25512154

10. Park JY, von Karsa L, Herrero R. Prevention strategies for gastric cancer: a global perspective. Clin Endosc. 2014; 47(6):478–89. https://doi.org/10.5946/ce.2014.47.6.478 PMID: 25505712

11. Graham DY, Fischbach L. Helicobacter pylori treatment in the era of increasing antibiotic resistance. Gut. 2010; 59(8):1143–53. https://doi.org/10.1136/gut.2009.192757 PMID: 20525969

12. O’Connor A, Fischbach W, Gisbert JP, O’Morain C. Treatment of Helicobacter pylori infection 2016. Helicobacter. 2016; 21 Suppl 1:55–61.

13. Ha NC, Oh ST, Sung JY, Cha KA, Lee MH, Oh BH. Supramolecular assembly and acid resistance of Helicobacter pylori urease. Nat Struct Biol. 2001; 8(6):505–9. https://doi.org/10.1038/85563 PMID: 11373617

14. Hu LT, Foxall PA, Russell R, Mobley HL. Purification of recombinant Helicobacter pylori urease apoenzyme encoded by ureA and ureB. Infect Immun. 1992; 60(7):2657–66. PMID: 1612735

15. Mobley HLT. Urease. In: Mobley HLT, Mendz GL, L. HS, editors. Helicobacter pylori- genes essential for gastric colonization. J Exp Med. 2003; 197(7):813–22. https://doi.org/10.1084/jem.20021531 PMID: 12668646

16. Bauerfeind P, Garner R, Dunn BE, Mobley HL. Synthesis and activity of Helicobacter pylori urease and catalase at low pH. Gut. 1997; 40(1):25–30. PMID: 9155571

17. Andritus KA, Fox JG, Schauer DB, Marini RP, Murphy JC, Yan L, et al. Inability of an isogenic urease-negative mutant strain of Helicobacter mustelae to colonize the ferret stomach. Infect Immun. 1995; 63(9):3722–5. PMID: 7642314

18. Eaton KA, Krakowska S. Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by Helicobacter pylori. Infect Immun. 1994; 62(9):3604–7. PMID: 8063376

19. Kavermann H, Burns BP, Angermuller K, Odenbreit S, Fischer W, Melchers K, et al. Identification and characterization of Helicobacter pylori genes essential for gastric colonization. J Exp Med. 2003; 197(7):813–22. https://doi.org/10.1084/jem.20021531 PMID: 12668646

20. Tsuda M, Karita M, Morshed MG, Okita K, Nakazawa T. A urease-negative mutant of Helicobacter pylori constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. Infect Immun. 1994; 62(8):3586–9. PMID: 8039935

21. Wirth HP, Beins MH, Yang M, Tham KT, Blaser MJ. Experimental infection of Mongolian gerbils with wild-type and mutant Helicobacter pylori strains. Infect Immun. 1998; 66(10):4856–66. PMID: 9746590

22. Lytton SD, Fischer W, Nagel W, Haas R, Beck FX. Production of ammonium by Helicobacter pylori mediates occludin processing and disruption of tight junctions in Caco-2 cells. Microbiology. 2005; 151(Pt 10):3267–76. https://doi.org/10.1099/mic.0.28049-0 PMID: 16207910

23. Wroblewski LE, Shen L, Ogden S, Romero-Gallo J, Lapiere LA, Israel DA, et al. Helicobacter pylori dysregulation of gastric epithelial tight junctions by urease-mediated myosin II activation. Gastroenterology. 2009; 136(1):236–46. https://doi.org/10.1053/j.gastro.2008.10.011 PMID: 18996125

24. Kuwahara H, Miyamoto Y, Akaife T, Kubota T, Sawa T, Okamoto S, et al. Helicobacter pylori urease suppresses bactericidal activity of peroxynitrite via carbon dioxide production. Infect Immun. 2000; 68(8):4378–83. PMID: 10899833

25. Fan X, Gunashe S, Cheng Z, Espejo R, Crowe SE, Ernst PB, et al. Helicobacter pylori urease binds to class II MHC on gastric epithelial cells and induces their apoptosis. J Immunol. 2000; 165(4):1918–24. PMID: 10925273

26. Beswick EJ, Pinchuk IV, Minch K, Suarez G, Sierra JC, Yamaoka Y, et al. The Helicobacter pylori urease B subunit binds to CD74 on gastric epithelial cells and induces NF-kappaB activation and interleukin-8 production. Infect Immun. 2006; 74(2):1148–55. https://doi.org/10.1128/IAI.74.2.1148-1155.2006 PMID: 16428763

27. Harris PR, Mobley HL, Perez-Perez GI, Blaser MJ, Smith PD. Helicobacter pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. Gastroenterology. 1996; 111(2):419–25. PMID: 8690207
28. Schoep TD, Fulurija A, Good F, Lu W, Himенеz RP, Schwan C, et al. Surface properties of *Helicobacter pylori* urease complex are essential for persistence. PLoS One. 2010; 5(11):e15042. https://doi.org/10.1371/journal.pone.0015042

29. Williams CL, Preston T, Hossack M, Slater C, McColl KE. *Helicobacter pylori* utilises urea for amino acid synthesis. FEMS Immunol Med Microbiol. 1996; 13(1):87–94. PMID: 8821403

30. Bertram R, Hillen W. The application of Tet repressor in prokaryotic gene regulation and expression. Microbiol Biotechnol. 2008; 1(1):2–16. https://doi.org/10.1111/j.1751-7915.2007.00001.x

31. Berens C, Hillen W. Gene regulation by tetracyclines. Constraints of resistance regulation in bacteria shape TetR for application in eukaryotes. Eur J Biochem. 2003; 270(15):3109–21. PMID: 12869186

32. Debowski AW, Verbrugghe P, Sehnal M, Marshall BJ, Benghezal M. Development of a tetracycline-inducible gene expression system for the study of *Helicobacter pylori* pathogenesis. Appl Environ Microbiol. 2013; 79(23):7351–9. https://doi.org/10.1128/AEM.02701-13 PMID: 24056453

33. Labigne A, Cussac V, Courcoux P. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. J Bacteriol. 1991; 173(6):1920–31. PMID: 2001995

34. Davies BJ, de Vries N, Rijpkema SG, van Vliet AH, Penn CW. Transcriptional and mutational analysis of the *Helicobacter pylori* urease promoter. FEMS Microbiol Lett. 2002; 213(1):27–32. PMID: 1217484

35. Gandotra S, Schnappinger D, Monteleone M, Hillen W, Ehrt S. In vivo gene silencing identifies the *Mycobacterium tuberculosis* proteasome as essential for the bacteria to persist in mice. Nat Med. 2007; 13(12):1515–20. https://doi.org/10.1038/nm1683 PMID: 18059281

36. Schonig K, Bujard H, Gossen M. The power of reversibility regulating gene activities via tetracycline-controlled transcription. Methods Enzymol. 2010; 477:429–53. https://doi.org/10.1016/S0076-6879(10)77022-1 PMID: 20699154

37. Ehrt S, Guo XY, Hickey CM, Ryou M, Monteleone M, Riley LW, et al. Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. Nucleic Acids Res. 2005; 33(2):e21. https://doi.org/10.1093/nar/gni013 PMID: 15687379

38. Maier RJ, Benoit SL, Seshadri S. Nickel-binding and accessory proteins facilitating Ni-enzyme maturation in *Helicobacter pylori*. Biometals. 2007; 20(3–4):655–64. https://doi.org/10.1007/s10534-006-0961-8 PMID: 17205208

39. Stingl K, De Reuse H. Staying alive overdosed: how does *Helicobacter pylori* control urease activity? Int J Med Microbiol. 2005; 295(5):307–15. https://doi.org/10.1016/j.ijmm.2005.06.006 PMID: 16173497

40. van Vliet AH, Poppelaars SW, Davies BJ, Stooj J, Bereswill S, Kist M, et al. NikR mediates nickel-responsive transcriptional induction of urease expression in *Helicobacter pylori*. Infect Immun. 2002; 70(6):2846–52. https://doi.org/10.1128/IAI.70.6.2846-2852.2002 PMID: 12010971

41. Centlivre M, Zhou X, Pouw SM, Weijer K, Kleibeuker W, Das AT, et al. Autorregulatory lentiviral vectors allow multiple cycles of doxycycline-inducible gene expression in human hematopoietic cells in vivo. Gene Ther. 2010; 17(1):14–25. https://doi.org/10.1038/gt.2009.109 PMID: 19727135

42. Ludwig A, Schlierb B, Scharadt A, Nave KA, Wegner M. Sox10-tTA mouse line for tetracycline-inducible gene expression system for the study of *Helicobacter pylori* infection. Appl Environ Microbiol. 2013; 79(23):7351–9. https://doi.org/10.1128/AEM.02701-13 PMID: 24056453

43. Kraft C, Suerbaum S. Mutation and recombination in *Helicobacter pylori* mechanims and role in generating strain diversity. Int J Med Microbiol. 2005; 295(5):299–305. https://doi.org/10.1016/j.ijmm.2005.06.002 PMID: 16173496

44. Garcia-Ortiz MV, Marsin S, Arana ME, Gasparutto D, Gueros R, Kunkel TA, et al. Unexpected role for *Helicobacter pylori* DNA polymerase I as a source of genetic variability. PLoS Genet. 2011; 7(6):e1002152. https://doi.org/10.1371/journal.pgen.1002152 PMID: 21731507

45. Orillard E, Radicella JP, Marsin S. Biochemical and cellular characterization of *Helicobacter pylori* RecA, a protein with high-level constitutive expression. J Bacteriol. 2011; 193(23):6490–7. https://doi.org/10.1128/JB.05646-11 PMID: 21949074

46. Krebes J, Dideot X, Kennemann L, Suerbaum S. Bidirectional genomic exchange between *Helicobacter pylori* strains from a family in Coventry, United Kingdom. Int J Med Microbiol. 2014; 304(8):1135–46. https://doi.org/10.1016/j.ijmm.2014.08.007 PMID: 25218701

47. Linz B, Windsor HM, McGraw JJ, Hansen LM, Gajewski JP, Tomsho LP, et al. A mutation burst during the acute phase of *Helicobacter pylori* infection in humans and rhesus macaques. Nat Commun. 2014; 5:4165. https://doi.org/10.1038/ncomms5165 PMID: 24924186

48. Hecht B, Muller G, Hillen W. Noninducible Tet repressor mutations map from the operator binding motif to the C terminus. J Bacteriol. 1993; 175(4):1206–10. PMID: 8432715
49. Muller G, Hecht B, Helbl V, Hinrichs W, Saenger W, Hillen W. Characterization of non-inducible Tet repressor mutants suggests conformational changes necessary for induction. Nat Struct Biol. 1995; 2 (8):693–703. PMID: 7552732

50. Scholz O, Henssler EM, Bail J, Schubert P, Bogdanska-Urbaniak J, Sopp S, et al. Activity reversal of Tet repressor caused by single amino acid exchanges. Mol Microbiol. 2004; 53(3):777–89. https://doi.org/10.1111/j.1365-2958.2004.04159.x PMID: 15255892

51. Josenhans C, Niehus E, Amersbach S, Horster A, Betz C, Drescher B, et al. Functional characterization of the antagonist flagellar late regulators FljA and FlgM of Helicobacter pylori and their effects on the H. pylori transcriptome. Mol Microbiol. 2002; 43(2):307–22. PMID: 11958711

52. Niehus E, Gressmann H, Ye F, Schlapbach R, Dehio M, Dehio C, et al. Genome-wide analysis of transcriptional hierarchy and feedback regulation in the flagellar system of Helicobacter pylori. Mol Microbiol. 2004; 52(4):947–61. https://doi.org/10.1111/j.1365-2958.2004.04006.x PMID: 15130117

53. Lertsethakarn P, Ottemann KM, Hendrixson DR. Motility and chemotaxis in Campylobacter and Helicobacter. Annu Rev Microbiol. 2011; 65:389–410. https://doi.org/10.1146/annurev-micro-090110-102908 PMID: 21939377

54. Tsang J, Hoover TR. Basal Body Structures Differentially Affect Transcription of RpoN- and FliA-Dependent Flagellar Genes in Helicobacter pylori. J Bacteriol. 2015; 197(11):1921–30. https://doi.org/10.1128/JB.02533-14 PMID: 25825427

55. Eaton KA, Suerbaum S, Josenhans C. Colonization of gnotobiotic piglets by Helicobacter pylori: Annu Rev Microbiol. 2011; 65:389–410. https://doi.org/10.1146/annurev-micro-090110-102908 PMID: 21939377

56. Ottemann KM, Lowenthal AC. Helicobacter pylori uses motility for initial colonization and to attain robust infection. Infect Immun. 2002; 70(4):1984–90. https://doi.org/10.1128/IAI.70.4.1984-1990.2002 PMID: 11895962

57. Schreiber S, Konradt M, Groll C, Scheid P, Hanauer G, Werling HO, et al. The spatial orientation of Helicobacter pylori in the gastric mucus. Proc Natl Acad Sci U S A. 2004; 101(14):5024–9. https://doi.org/10.1073/pnas.0308386101 PMID: 15044704

58. Suerbaum S, Josenhans C. Helicobacter pylori evolution and phenotypic diversification in a changing host. Nat Rev Microbiol. 2007; 5(6):441–52. https://doi.org/10.1038/nrmicro1658 PMID: 17505524

59. Celli JP, Turner BS, Afdhal NH, Keates S, Ghiran I, Kelly CP, et al. Urease is essential for chronic H. pylori infection. Infect Immun. 2002; 70(4):1984–90. https://doi.org/10.1128/IAI.70.4.1984-1990.2002 PMID: 21939377

60. Styer CM, Hansen LM, Cooke CL, Gundersen AM, Choi SS, Berg DE, et al. Expression of the BabA adhesin during experimental infection with Helicobacter pylori. Infect Immun. 2010; 78(4):1593–600. https://doi.org/10.1128/IAI.01297-09 PMID: 20123715

61. Daliliene D, Daillde G, Kersulyte D, Berg DE. Contraselectable streptomycin susceptibility determinant for genetic manipulation and analysis of Helicobacter pylori. Appl Environ Microbiol. 2006; 72(9):5908–14. https://doi.org/10.1128/AEM.01135-06 PMID: 16957210

62. Chaiker AF, Minehart HW, Hughes NJ, Koretk KA, Brinkman KK, et al. Systematic identification of selective essential genes in Helicobacter pylori by genome prioritization and allelic replacement mutagenesis. J Bacteriol. 2001; 183(4):1259–68. https://doi.org/10.1128/JB.183.4.1259-1268.2001 PMID: 11157938

63. Shevchuk NA, Bryksin AV, Nusinovich YA, Cabello FC, Sutherland M, Ladisch S. Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. Nucleic Acids Res. 2004; 32(2):e19. https://doi.org/10.1093/nar/gnh014 PMID: 14739232

64. Chua EG, Wise MJ, Khosravi Y, Seow SW, Amoyo AA, Pettersson S, et al. Quantum changes in Helicobacter pylori gene expression accompany host-adaptation. DNA research: an international journal for rapid publication of reports on genes and genomes. 2013. Nucleic Acids Res. 2013; 41(Database issue):D387–95. https://doi.org/10.1093/nar/gks1234 PMID: 23197656
Urease is essential for chronic *H. pylori* infection

69. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014; 30(14):2068–9. https://doi.org/10.1093/bioinformatics/btu153 PMID: 24642063

70. Wilson D, Madera M, Vogel C, Chothia C, Gough J. The SUPERFAMILY database in 2007: families and functions. Nucleic Acids Res. 2007; 35(Database issue):D308–13. https://doi.org/10.1093/nar/gkl910 PMID: 17098927

71. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012; 28(12):1647–9. https://doi.org/10.1093/bioinformatics/bts199 PMID: 22543367

72. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nature methods. 2012; 9(4):357–9. https://doi.org/10.1038/nmeth.1923 PMID: 22388286