The development of genetic tools to study *Helicobacter pylori* pathogenesis and persistence.

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All work presented in this thesis was performed by myself, unless stated otherwise.

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AW Debowski contributed to research design, experimental work, data analysis and writing of the manuscript. A.W. Debowski’s overall contribution to the paper: 65 %.

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Summary

*Helicobacter pylori* is an ancient member of the human microbiota that has coevolved with humans to dominate the gastric niche. Infection causes chronic active gastritis which may develop into peptic ulceration or more rarely gastric adenocarcinoma, however, the majority of infected individuals (80-90%) carry and transmit *H. pylori* without any symptoms of disease. Furthermore, there is mounting epidemiological and recent experimental evidence that suggest that *H. pylori* infection may be protective against immune diseases.

A limited number of genetic tools are available to study *H. pylori* pathogenesis. In particular, gene expression systems, that allow the regulation of bacterial genes during an infection are lacking. Such a genetic tool is of particular importance to study the functional role and temporal requirements of *H. pylori* virulence determinants as infection is persistent and clinical diseases develop after many years of chronic inflammation and epithelial damage. This study describes the development of chromosomal *H. pylori* gene regulation systems based on tetracyclines for the control of gene expression, both *in vitro* and *in vivo*. These systems are based on the *Escherichia coli* Tn10-derived tet-regulatory system and consist of strong tet operator (tetO) containing *H. pylori* promoters, expression cassettes for tet repressors, TetR and revTetR, and the chemical effectors ATc and Dox.

Using GFP as a reporter gene, this study demonstrated that TetR-controlled gene expression was effector concentration-dependent and maximal with ATc concentrations 10-fold below the minimal inhibitory concentration for *H. pylori*. Regulatory windows could be adjusted by changing tet-promoter and TetR expression cassette combinations. Furthermore, using conditional GFP expressing *H. pylori* mutants, this study demonstrated gene expression could be regulated in an animal host using the chromosome based tet-system, demonstrating that tet-regulation can be used to study of a range of different *H. pylori* genes during chronic infection.

Using the essential colonization factor urease, this study showed that these tet-expression systems can be used to construct conditional knockouts of essential colonization factors to analyse gene function during the chronic phase of *H. pylori*
Summary

infection in mice. Mouse infection experiments using tet-conditional X47 urease mutants conducted in this study provided the first conclusive evidence that urease is required to establish and maintain infection and furthermore that \textit{H. pylori} is subjected to strong selective pressures to continuously express urease in order to survive in its gastric niche.

This study also showed that \textit{H. pylori} cholesterol \textalpha{}-glucosyltransferase (CGT), an enzyme implicated in immune modulation, was expressed in very low abundance and that perturbations in CGT expression, induced by \textit{tet}-regulation, affected the ability of \textit{H. pylori} to establish infection. This data suggests that the controlled expression of CGT by \textit{H. pylori} is important for the bacterium’s fitness \textit{in vivo}.

The efficacy of four chromosomal loci, \textit{mdaB}, \textit{gltDH}, \textit{trpA} and \textit{dapB} locus, as suitable for chromosomal complementation systems was also demonstrated. These regions supported the expression of foreign proteins with no discernible impact on the colonization fitness of strain X47. Lastly, this study also involved the characterization of the \textit{dif}/XerH system in \textit{H. pylori} which facilitated the adaptation of Xer-cision as a new genetic tool for the expedited generation of markerless \textit{H. pylori} mutants. The chromosomal based \textit{tet}-system and “Xer-cise” technology developed in this study should be transferable to a large variety of \textit{H. pylori} isolates with different genetic backgrounds.
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## Abbreviations

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<tbody>
<tr>
<td>βCG</td>
<td>cholesteryl β-D-glucopyranoside</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>aa</td>
<td>amino acids</td>
</tr>
<tr>
<td>amiE</td>
<td>acylamide amidohydrolase gene</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>ampicillin resistant</td>
</tr>
<tr>
<td>ArsR&lt;~P&gt;</td>
<td>phosphorylated acid responsive signalling response regulator</td>
</tr>
<tr>
<td>ArsRS</td>
<td>acid responsive signalling two-component system</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base-pair</td>
</tr>
<tr>
<td>cat</td>
<td>chloramphenicol resistant gene</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming units</td>
</tr>
<tr>
<td>clI</td>
<td>phage lambda cl repressor protein</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;/Cm&lt;sup&gt;s&lt;/sup&gt;</td>
<td>Chloramphenicol resistant/chloramphenicol sensitive</td>
</tr>
<tr>
<td>d</td>
<td>days</td>
</tr>
<tr>
<td>dapB</td>
<td>dihydrodipicolinate reductase gene</td>
</tr>
<tr>
<td>dif</td>
<td>deletion induced filamentation sequence</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dox</td>
<td>doxycycline</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>flaA</td>
<td>flagellin subunit A gene</td>
</tr>
<tr>
<td>FtsK</td>
<td>filamenting temperature sensitive protein K</td>
</tr>
<tr>
<td>ftsK</td>
<td>filamenting temperature sensitive gene K</td>
</tr>
<tr>
<td>G</td>
<td>gauge</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
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Abbreviations

\begin{itemize}
\item \textit{g} \quad \text{relative centrifugal force}
\item \textit{GFP} \quad \text{green fluorescent protein}
\item \textit{gfp} \quad \text{green fluorescent protein gene}
\item \textit{gltDH} \quad \text{glutamate dehydrogenase gene}
\item \textit{h} \quad \text{hour}
\item \textit{HA} \quad \text{hemagglutinin}
\item \textit{IgA} \quad \text{Immunoglobulin A}
\item \textit{IgG} \quad \text{Immunoglobulin G}
\item \textit{Kan}\textsuperscript{r} \quad \text{kanamycin resistant}
\item \textit{kb} \quad \text{kilo base-pair}
\item \textit{kDa} \quad \text{kilodalton}
\item \textit{l} \quad \text{litre}
\item \textit{Le}\textsuperscript{+} \quad \text{Lewis blood group antigen positive}
\item \textit{Le}\textsuperscript{-} \quad \text{Lewis blood group antigen negative}
\item \textit{M} \quad \text{molar}
\item \textit{mdaB} \quad \text{modulator of drug activity gene}
\item \textit{mg} \quad \text{milligram}
\item \textit{MIC} \quad \text{minimal inhibitory concentration}
\item \textit{min} \quad \text{minute}
\item \textit{ml} \quad \text{millilitre}
\item \textit{mM} \quad \text{millimolar}
\item \textit{mm} \quad \text{millimetre}
\item \textit{mRNA} \quad \text{messenger RNA}
\item \textit{MW} \quad \text{molecular weight}
\item \textit{NADPH} \quad \text{nicotinamide adenine dinucleotide phosphate}
\item \textit{NaOAc} \quad \text{sodium acetate}
\item \textit{NEB} \quad \text{New England Biolabs}
\item \textit{ng} \quad \text{nanogram}
\item \textit{NikR} \quad \text{nickel-responsive regulator protein}
\item \textit{nm} \quad \text{nanometer}
\item \textit{nt} \quad \text{nucleotide}
\item \textit{OD}_{600} \quad \text{optical density at 600 nm}
\item \textit{ORF} \quad \text{open reading frame}
\item \textit{ori} \quad \text{origin of replication}
\item \textit{pfr} \quad \text{non-haem iron-containing ferritin gene}
\end{itemize}
Abbreviations

PMSF  phenylmethanesulfonyl fluoride

rdxA  NADPH nitroreductase gene

Rf  retardation factor

RFU  relative fluorescence unit

rpm  revolutions per minute

rpsL  30s ribosomal protein S12 gene

s  second

sps  species

SOC  super optimal broth with glucose

Strr/Strs  streptomycin resistant/streptomycin sensitive

tet  tetracycline dependent

TetR  tetracycline resistance

T/H  T helper cell

TLC  thin layer chromatography

trpA  tryptophan synthase subunit alpha gene

U  unit (enzyme unites)

USA  United States of America

UV  ultra violet

UWA  University of Western Australia

V  volts

v/v  volume/volume ratio

wks  weeks

w/v  weight/volume ratio

Xer  chromosomal ColE1 recombination function/s protein

xer  chromosomal ColE1 recombination function/s gene
1 Literature review

The human body is colonized by a myriad of different microorganisms, which outnumber human cells by 10:1 (Savage, 1977; Sekirov et al., 2010). Most of these microorganisms are beneficial, forming complex communities (Andersson et al., 2008) that aid the extraction of nutrients from our food, synthesize essential vitamins and growth factors (Hooper et al., 2002), promote the development and function of mucosal tissue, and stimulate and direct the maturation of the immune system (Gordon and Pesti, 1971; Pamer, 2007; Rakoff-Nahoum et al., 2004; Sekirov et al., 2010; Smith et al., 2007). However some microorganisms that enter the body are not beneficial, instead they cause a variety of diseases that in some instances result in death.

*Helicobacter pylori* is one microorganism that may be considered both as a pathogen and as a beneficial symbiont. *H. pylori* are highly motile spiral-shaped gram-negative bacteria that colonize the human stomach (Marshall and Warren, 1984; Warren and Marshall, 1983). *H. pylori* is defined as a pathogen because infection causes chronic active gastritis which may develop into peptic ulceration or, more rarely, gastric adenocarcinoma (Atherton, 2006; Kusters et al., 2006). However, this ancient member of the human microbiota has coevolved with humans to dominate the gastric niche (Andersson et al., 2008; Bik et al., 2006; Linz et al., 2007) and the majority of infected individuals (80-90%) carry and transmit *H. pylori* without any symptoms of disease. Furthermore, there is mounting epidemiological and recent experimental evidence that suggest that *H. pylori* infection is protective against immune diseases such as childhood asthma, allergic rhinitis and skin allergies (Arnold et al., 2011; Blaser et al., 2008; Chen and Blaser, 2008; Codolo et al., 2008; Oertli et al., 2012). Understanding how and when colonization by this bacterium is beneficial and why some infections result in disease is critical to a better understanding of the role of microbiota in human health and disease as a whole. This knowledge has important applications in developing strategies to improve quality of life.
1.1 *Helicobacter pylori*: a general introduction

1.1.1 Epidemiology of *H. pylori* infection and disease

*H. pylori* has the highest prevalence of any human pathogen, colonizing approximately half the world’s population (Group, 1993). Infection is usually acquired during early childhood and can persist for the individual’s lifetime if untreated (Everhart, 2000; Kuipers et al., 1995). Though ubiquitous in all human populations, the prevalence of *H. pylori* infection shows large geographical variations. Generally, in developing countries more than 80% of the population is *H. pylori* positive while the prevalence of *H. pylori* in industrialised countries is much lower, ranging between 20% and 40% (Bruce and Maaroos, 2008). *H. pylori* prevalence can also vary within geographic regions, increasing proportionally with low socioeconomic conditions, poor levels of sanitation and overcrowding (Marshall and Windsor, 2005). The global prevalence of *H. pylori* infection is steadily declining, especially in developed countries, due to improved socioeconomic conditions and the active elimination of carriership, by way of antimicrobial treatment, which all act to reduce the chances of childhood infection (Bruce and Maaroos, 2008; Goh et al., 2011).

The exact mode of *H. pylori* transmission remains unclear due to the difficult and inconsistent isolation of *H. pylori* from environmental sources or niches external to the gastric environment (Goh et al., 2011; Khalifa et al., 2010; Kusters et al., 2006). Humans are the natural host for *H. pylori* (Amieva and El-Omar, 2008) and epidemiologic studies of *H. pylori* transmission show that the majority of infections tend to occur within families (Kivi et al., 2005; McCallion et al., 1996; Perry et al., 2006; Weyermann et al., 2006). Since the bacterium is extremely sensitive to atmospheric oxygen pressure and has a narrow temperature tolerance (34-40 °C) the most likely route of *H. pylori* transmission is believed to be through direct person-to-person contact (Amieva and El-Omar, 2008; Kusters et al., 2006). However, several studies have found positive correlations between *H. pylori* prevalence and the consumption of contaminated food or water and therefore environmental reservoirs must still be considered as possible sources of infection (Goh et al., 2011; Khalifa et al., 2010).
*H. pylori* infection is the main cause of peptic ulceration and is associated with the development of gastric mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma (Blaser and Atherton, 2004; Marshall and Warren, 1984; Parsonnet et al., 1994; Peek and Blaser, 2002). Gastric colonization with *H. pylori* induces chronic active gastritis in all infected individuals, however only a minority develop any clinical disease (Amieva and El-Omar, 2008; Kusters et al., 2006). Infected individuals have a 10-20% life-time risk of developing ulcer disease after many years of infection and inflammation. It has been estimated that *H. pylori* infection increases the risk of gastric cancer approximately 10-fold, meaning that infected individuals have a 1-2% risk of developing distal gastric cancer after several decades of chronic inflammation and tissue damage. Consequently, the World Health Organizations (WHO) has classified *H. pylori* as a class I carcinogen (Cancer, 1994). However, the risk of development of these gastric disorders in the presence of *H. pylori* infection depends on a variety of bacterial, host and environmental factors. (Kusters et al., 2006)

The successful isolation and *in vitro* culture of *H. pylori* from human gastric mucosal specimens facilitated the identification of *H. pylori* infection, rather than stress or spicy food as had been assumed before, as the cause of most peptic ulcers and gastritis (Marshall and Warren, 1984). Since this discovery, significant advances have been made in our understanding of *H. pylori* and, more importantly, the function of the human mucosal immune system.

### 1.1.2 *H. pylori* challenges to establishing chronic infection

There are several challenges that *H. pylori* encounters and has to overcome in order to first colonise and establish itself in the gastric niche, and then to maintain infection and avoid clearance by the host immune system to persist as a lifelong infection (Figure 1-1).

#### 1.1.2.1 Survival in the highly acidic gastric environment

The human stomach is a very hostile environment. The low pH of the gastric lumen is sufficient to either kill or inhibit the growth of most ingested microorganisms, and thus for many decades it was believed that microorganisms could not thrive in the gastric environment (Giannella et al., 1972; Husebye, 2005).
Chapter 1: Literature review

Figure 1-1 Colonization of the gastric niche

*H. pylori* enters the stomach and is attacked by proteases and acid. The activity of urease and other acid resistance mechanisms buy the bacteria precious time to quickly escape the lumen into the protective mucus layer. Bacterial flagella and chemotactic signalling systems allow the bacterium to swim in the neutral region of the mucus layer near the epithelial cell surface. *H. pylori* uses several adhesins to interact with epithelial cells to avoid clearance from the mucosa, sequester cholesterol and deliver bacterial toxins such as VacA and CagA. *H. pylori* contact with epithelial cells and toxin secretion induce tissue damage and the release of proinflammatory cytokines. Dendritic cells, monocytes and T cells are recruited to the site of infection and in turn secrete a number of cytokines and chemokines, resulting in typical gastritis. (Adapted from Fischer et al., 2009)

The first hurdle for *H. pylori* infection is surviving exposure to the low pH of the gastric lumen upon entry into the stomach. *H. pylori* is a neutralophile and as such cannot survive when the pH falls below 4.0, however *H. pylori* does have several resistance mechanisms that allow it to survive long enough to reach its niche, the mucus lining that protects the stomach tissue from its own acidic juices. The most important factor to *H. pylori* acid resistance is the abundant expression of a highly active enzyme known as urease. Urease transiently buffers the acidic environment around the bacteria by generating ammonia from urea hydrolysis (Discussed in further detail in Chapter 6). *H. pylori* also possesses urease independent mechanisms to adapt to mild acidic
conditions and uses several stress responses to transiently withstand acid (Merrell et al., 2003; Pflock et al., 2006). Furthermore, *H. pylori* membrane proteins have modifications that increase their isoelectric points (~8.9) compared to their homologous counterparts in other neutralophiles (6.3) which reduces proton influx across the inner and outer membranes, facilitating buffering of the periplasm by urease generated ammonia (Sachs et al., 2003). Despite its array of acid resistance mechanisms, *H. pylori* can only survive for a few minutes in the gastric lumen (Schreiber et al., 2005). Therefore *H. pylori* is also equipped with multiple polar flagella and chemoreceptors which allow it to quickly orientate in the gastric lumen and swim to the mucus layer covering the epithelium where the pH values are more neutral.

1.1.2.2 Staying in the safety zone

*H. pylori* has evolved several strategies to minimize exposure to the low pH in the stomach lumen by remaining in close proximity to the surface of the epithelium where the pH is near neutral.

Motility and chemotaxis play key roles in infection establishment (Croxen et al., 2006; McGee et al., 2005; Ottemann and Lowenthal, 2002; Terry et al., 2005). To remain in the mucus layer, and avoid clearance from the gut due to constant and rapid turnover of the mucus lining, *H. pylori* has up to six polar flagella that allow the bacteria to swim through the viscous mucus lining and uses chemotactic signalling systems to control the direction of its movements (Rust et al., 2008). Most *H. pylori* are free swimming within the narrow band of the protective mucus gel, actively staying within 25 μm of the epithelial cell surface, and avoid the acidic distal regions by orientating themselves based on the pH gradient in the mucus layer (Schreiber et al., 2004). Approximately 20% of the *H. pylori* population in the stomach are found adhered to surface of epithelial cells and some bacteria are found deeper in the gastric tissue or even in the intercellular spaces (Camorlinga-Ponce et al., 2004; Dubois and Boren, 2007; Necchi et al., 2007).

Adherence to the gastric mucosa is widely assumed to play an important role in the initial colonization and long-term persistence of *H. pylori* in the human gastric mucosa. *H. pylori* has several outer membrane proteins of the Hop (*Helicobacter outer membrane protein*) family that act as adhesins and allow the bacteria to adhere to the epithelial cell surface (Yamaoka and Alm, 2008). The best studied adhesins are the
blood group antigen binding adhesin (BabA, also known as HopS) and sialic acid binding adhesin (SabA, also known as HopP) which recognize and bind to specific carbohydrate motifs on host glycoproteins. BabA binds to fucosylated blood group antigen Lewis b (Le^b) and related fucosylated ABO blood group antigens (Ilver et al., 1998; Yamaoka and Alm, 2008). Le^b is present on both epithelial cells and on mucus glycoproteins, meaning that BabA not only mediates adherence to the gastric mucosa but may also mediate binding within the mucus layer in a pH dependent manner (Linden et al., 2004; Linden et al., 2002). SabA mainly recognizes the sialyl-Lewis x (sLe^x) antigen but may also bind to sialyl-Lewis a (sLe^a) and other sialylated glycoproteins (Aspholm et al., 2006; Mahdavi et al., 2002). SabA may function to replace adhesion through BabA in inflamed tissues as prolonged gastric inflammation results in replacement of non-sialylated carbohydrate motifs with sialylated Lewis antigens (Mahdavi et al., 2002). Other outer membrane proteins identified to be involved in adhesion are the adherence-associated lipoproteins, AlpA and AlpB (also known as HopC and HopB respectively), the outer inflammatory protein OipA (also known as HopH) and HopZ, however the receptors recognized by these adhesins remain to be identified (Backert et al., 2011; Yamaoka and Alm, 2008). Apart from outer membrane proteins, adhesion to the gastric mucosa may also be mediated by lipopolysaccharide (LPS). *H. pylori* LPS contains many different Lewis antigens and the composition of LPS can be dramatically varied by phase-variable expression of the different genes for LPS synthesis (Moran and Trent, 2008). The presence of Le^x antigens in *H. pylori* LPS has been shown to mediate binding to galectin-3, a lectin on the epithelial cell surface which is upregulated by the action of *H. pylori* toxins (Edwards et al., 2000; Fowler et al., 2006).

No individual adhesin is essential for attachment to the gastric mucosa. Expression of individual adhesins is diverse between strains and can be variable within a single strain over the period of infection (de Jonge et al., 2004; Dossumbekova et al., 2006; Solnick et al., 2004; Styer et al., 2010). This dynamic variability, in the binding affinity and specificity of individual adhesins, may be necessary for *H. pylori* to adapt to changes in mucosal glycosylation within different regions of the gastric mucosa over time (Aspholm et al., 2006). The mechanisms of variability and adaptation work on a genetic level through on/off switching of gene expression, gene inactivation and by allelic recombination (Yamaoka and Alm, 2008). Adherence to the gastric mucosa likely mediates persistent infection through several mechanisms. Adhesive interactions help
avoid mechanical clearance from the stomach, contribute to inflammation and are also likely involved in disease progression (Yamaoka and Alm, 2008). Adhesion may also provide *H. pylori* with the opportunity to sequester cholesterol from host cells and to deliver bacterial toxins, which cause inflammation and tissue damage to release essential nutrients and ions into the nutrient-poor gastric mucin layer (Fischer et al., 2009).

### 1.1.2.3 Evading clearance by host defences

A robust immune response is generated against *H. pylori*, in terms of inflammation and the production of both serum and mucosal antibodies. However, *H. pylori* is able to remain in the stomach mucosa for many decades if the infection is not treated with antibiotics, indicating that the host immune response is ineffective (Fischer et al., 2009; Kusters et al., 2006; Wilson and Crabtree, 2007). *H. pylori* virulence factors have been shown to elicit both proinflammatory and immunosuppressive effects and thereby *H. pylori* avoids an appropriate immune response which instead leads to a chronic infection and mild inflammation or gastritis (Amieva and El-Omar, 2008; Beigier-Bompadre et al., 2011).

*H. pylori* uses a number of mechanisms to avoid recognition by the innate immune system. Host cells have several pattern recognition receptors (PRRs) which play important roles in initiating the innate immune response by recognizing molecules that are unique to, and broadly shared by, many microbial pathogens. One group of PPRs are the Toll-like receptors (TLRs). TLR5 recognises a highly conserved region within bacterial flagellin, a core component of the flagella required for motility. The flagellin of *H. pylori* and *Campylobacter jejuni* has evolved to become divergent in this region to prevent recognition by TLR5 (Andersen-Nissen et al., 2005; Galkin et al., 2008). TLR4 recognizes lipid A, the conserved molecular pattern of structurally diverse LPS molecules of gram-negative bacteria. *H. pylori* LPS however contains a lipid A domain that has a unique chemical structure which significantly reduces its recognition by TLR4 and thus shows up to 1,000 times lower immunological activity than lipid A of the Enterobacteriaceae family (Moran and Trent, 2008; Muotiala et al., 1992; Park et al., 2009). Furthermore, the unique structure of *H. pylori* lipid A also confers resistance to cationic antimicrobial peptides and has shown to be essential for colonization (Cullen et al., 2011; Tran et al., 2006).
H. pylori LPS is also unusual in that it usually contains carbohydrate motifs that mimic host glycan structures (Lewis blood group antigens) (Moran and Trent, 2008) to avoid detection by the host immune system. Lewis antigen expression in H. pylori is also highly phase variable as a result of on/off switching of the expression of different glycosyltransferase genes. This not only influences H. pylori adherence to epithelial cells (as discussed above) but also affects the binding interactions between H. pylori and dendritic cells (DCs). DCs express a receptor known as DC-SIGN (dendritic cell-specific intercellular adhesion molecule grabbing non-integrin) that recognises many pathogens, including H. pylori, and is involved in cell adhesion and antigen presentation to T cells (Bergman et al., 2004; Koppel et al., 2005). Binding of DC-SIGN is dependent on high-mannose glycans or non-sialylated Lewis antigens. H. pylori Le⁺ phase variants can bind DC-SIGN on DCs in the gastric mucosa and induce a shift in the T₁H₁/T₁H₂ balance towards T₁H₂-polarization, whereas Le⁻ phase variants escape binding to DCs and induce a strong T₁H₁ cell response (Bergman et al., 2004).

Interestingly, despite having mechanisms to avoid detection by the immune system, H. pylori also has several mechanisms by which it activates a strong response from the innate immune system and the bacterium exploits this response for its own purpose. The abundantly expressed H. pylori urease enzyme has been shown to cause cellular damage and inflammation, potentially promoting the release of essential nutrients into the gastric environment (Allen, 2000; Mobley et al., 1991). In addition, H. pylori produces an oligomeric protein called HP-NAP (H. pylori neutrophil-activating protein) which induces the recruitment of polymorphonuclear leukocytes (PMNs) to the lamina propria and it also has powerful neutrophil-activating activities. HP-NAP induces the secretion of cytokines from monocytes and neutrophils which may help to drive T₁H₁-polarization of the immune response (Allen, 2000; Amedei et al., 2006). Phagocytosis coupled with the production of reactive oxygen species (ROS) and nitric oxide (NO) are essential mechanisms of the immune system for the destruction of pathogens. HP-NAP stimulates NADPH oxidase assembly and the production of ROS by PMNs. To avoid the antimicrobial activity of ROS, H. pylori produces two detoxifying enzymes, catalase (Odenbreit et al., 1996b) and superoxide dismutase (Spiegelhalder et al., 1993). Phagocytised H. pylori also disrupt NADPH oxidase targeting, redirecting it to the cytoplasmic membrane where superoxide is consequently generated in the extracellular environment, promoting tissue damage, while the ingested bacteria remain unharmed in the phagosome (Allen et al., 2005). H. pylori also combats the production of
bactericidal NO. The expression of inducible nitric oxide synthase (iNOS) is activated both in *H. pylori* infected gastric tissues and in infected macrophages (Fu et al., 1999; Gobert et al., 2002b; Wilson et al., 1996). *H. pylori* inhibits the production of NO by expressing the enzyme arginase, RocF, which competes for the iNOS substrate, L-arginine, converting it to urea and L-ornithine instead of NO (McGee et al., 1999). In addition, *H. pylori* activates the expression of eukaryotic arginase II in macrophages which also competes with iNOS for L-arginine and induces macrophage apoptosis (Gobert et al., 2002a). Translation of iNOS is regulated by the availability of L-arginine, and therefore the combined arginase activities also result in the inhibition of iNOS expression, further facilitating *H. pylori* evasion of macrophage attack (Chaturvedi et al., 2007).

*H. pylori* has two major bacterial factors that are strongly associated with severe disease pathology. These are the certain genotypes of *vacA*, a gene encoding vacuolating cytotoxin A (VacA), and the presence of the cytotoxic-associated gene pathogenicity island (*cag*-PAI) (Fischer et al., 2009). The strong association between *H. pylori* strains carrying functional VacA and/or *cag*-PAI and an increased risk for gastric cancer has been well documented by numerous epidemiological studies and the reader is directed to excellent reviews for further reading on this subject (Hussein, 2010; Peek and Crabtree, 2006; Wen and Moss, 2009; Yamaoka et al., 2008). VacA is a multifunctional toxin secreted by *H. pylori* that displays numerous activities in different cell types (Cover and Blanke, 2005). VacA mediated effects on gastric epithelial cells include: stimulation of proinflammatory signalling, alterations in mitochondrial membrane permeability and apoptosis, increased permeability of the plasma membrane and alterations in endocytic compartments which ultimately results in the formation of characteristic cell vacuoles. The *cag*-PAI contains roughly 30 genes that encode the cytotoxic-associated gene A protein (CagA) and a type IV secretion system (T4SS) which is capable of translocating CagA from the bacteria to host gastric epithelial cells (Fischer, 2011). Once inside the eukaryotic cell, CagA interacts with a number of different target molecules and, depending on the *cagA* allele, these interactions can activate several signalling pathways that led to the disruption of cell-cell junctions, actin-cytoskeletal rearrangements, increased inflammation, and deregulation of the cell-division cycle (Tegtmeier et al., 2011). To date, CagA is the only effector protein known to be translocated by the Cag T4SS. However, the Cag T4SS has been implicated in the translocation of bacterial cell wall fragments leading to the activation
of the intracellular PRR, Nod-like receptor 1 (Nod1), in epithelial cells and the induction of proinflammatory chemokines (Viala et al., 2004).

Usually, if a microbial infection is not quickly resolved by the innate immune response, the adaptive immune response is activated and generates a specific, targeted response that resolves the infection (Murphy et al., 2008). However the adaptive response generated against \textit{H. pylori} is ineffective at clearing the infection and, following successful \textit{H. pylori} eradication by antibiotic treatment, it also fails to provide protection or memory against reinfection (Parsonnet, 2003). Transmission electron microscopy and immunogold detection on human stomach biopsies have shown that \textit{H. pylori} cells come into direct contact with immune cells of the lamina propria in the majority of cases of gastritis and gastric cancer (Necchi et al., 2007). \textit{H. pylori} has evolved complex mechanisms to interfere with many stages of the adaptive immune response, ranging from inhibition of antigen presentation to modulation of T cell cytokine signalling (Fischer et al., 2009). \textit{H. pylori} has a very unusual lipid composition, characterized by high concentrations of lysophospholipids and unique cholesteryl α-glucosides (Haque et al., 1995; Hirai et al., 1995). The presence of cholesteryl α-glucosides is thought to facilitate immune evasion by inhibiting phagocytosis by antigen presenting cells (APCs) and consequently limiting T cell activation (Wunder et al., 2006). The actions of urease and VacA have been shown to delay phagocytosis and inhibit phagosome maturation, permitting \textit{H. pylori} to persist inside large megasome structures and preventing further antigen processing for presentation to T cells (Schwartz and Allen, 2006; Zheng and Jones, 2003). \textit{H. pylori} also inhibits the proliferation of both B cells and T cells through the action of secreted bacterial factors VacA, γ-glutamyltranspeptidase (γGT) and arginase (Beigier-Bompadre et al., 2011; Gebert et al., 2003; Schmees et al., 2007; Torres et al., 2007; Zabaleta et al., 2004). These immune modulation mechanisms are thought to help \textit{H. pylori} achieve a delicate balance between stimulation of the immune system and suppression of effective inflammation so as to maintain chronic infection (Amieva and El-Omar, 2008; Beigier-Bompadre et al., 2011; Fischer et al., 2009).

\subsection*{1.1.3 The benefits of \textit{H. pylori} colonization}

Though the immune modulation capabilities of \textit{H. pylori} are designed to promote its own survival and persistence, these interactions may have significant impact on the
host’s immune response against other diseases. There have been several recent reports describing associations between *H. pylori* infection and less severe disease outcomes for gastroenteritis (Chang et al., 2011; Rothenbacher et al., 2000), and protection against reactivation of latent tuberculosis (Perry et al., 2010). More importantly, in many developed nations the decline in *H. pylori* infection rates has preceded the rise in the prevalence of asthma and other atopic diseases (Eder et al., 2006). Several case-control and cross-sectional studies have reported an inverse association of *H. pylori* infection with asthma, allergic rhinitis, and atopic dermatitis (Blaser et al., 2008). The negative association between the incidence of asthma and chronic infection with *H. pylori* was found to be strongest in young individuals with early-life asthma onset (Chen and Blaser, 2008) and was in part dependent on CagA (Chen and Blaser, 2007; Reibman et al., 2008). These observations have led to the formulation of the “disappearing microbiota” hypothesis (Blaser and Falkow, 2009), which postulates that the asthma and allergy epidemic of modern societies is a direct consequence of the disappearance of human ancestral indigenous microflora, which includes *H. pylori*. The recent development of a mouse model for allergic airway inflammation and hyper-responsiveness has provided some of the first experimental evidence in support of this hypothesis (Arnold et al., 2011; Oertli et al., 2012). These studies have shown *H. pylori* infection to be protective against allergen-induced asthma and propose that the mechanism for protection involves *H. pylori* mediated inhibition of proper DC maturation and *H. pylori* induced DC reprogramming toward a tolerance-promoting phenotype.

Evidently there are still many aspects of *H. pylori* infection that remain unclear and there is a great deal left to learn about *H. pylori* and its role as an ancient member of the human microbiome. Future studies directed towards understanding the interaction between *H. pylori* and immune cells *in vivo* are expected to result in important insights into the mechanisms of *H. pylori* persistence and the relationship between human microbiota and the immune system which seem to have important implications in human health and disease. To realise these insights, the development of new genetic tools that can be applied to the study of *H. pylori* is essential for advancing this important field of research.
1.2 Genetic tools used to gather our current knowledge

1.2.1 Genetic knockouts

The selective disruption of gene function has been, and remains, a critical element in elucidating information regarding gene essentiality for bacterial growth and/or pathogenesis. However, molecular genetic studies with *H. pylori* have been hampered compared to other well-known pathogens, such as *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus* and *Mycobacterium tuberculosis*, due to the limited number of appropriate genetic tools. One main reason for this is that the genetics of *H. pylori* is substantially different from that of *E. coli*, which is the model organism for bacterial genetics, and thus established genetic tools often do not work or require considerable optimization to adapt them to *H. pylori* (Boneca et al., 2008; Dailidiene et al., 2006; Kavermann et al., 2003; Odenbreit et al., 1996a). Therefore the construction of specific tools for the genetic manipulation of *H. pylori* remains an important task (Boneca et al., 2008; Heuermann and Haas, 1998).

Transposon mutagenesis is a powerful genetic tool for generating mutant libraries which can be screened to identify mutants that are absent and therefore represent essential genes or to identify mutants that are impaired in specific cellular functions, such as motility, chemotaxis or cell morphology, and so help define gene function (Bosse et al., 2006; Lehoux et al., 2001; Reznikoff and Winterberg, 2008). Unfortunately, *H. pylori* does not support the replication of common plasmid or transposon vectors and thus these systems have had to be tailored to *H. pylori* before their utility could be realised in full. The first mutant libraries were generated by shuttle mutagenesis (transposition of *H. pylori* DNA clones propagated in *E. coli*) (Hofreuter et al., 1998; Kavermann et al., 2003; Odenbreit et al., 1996a; Odenbreit et al., 1999) and later by the random insertion of suicide plasmids (Bijlsma et al., 1999). These first libraries, however, were still too small to be genome saturating. This limitation was finally overcome when advantage was taken of a newly developed *in vitro* mutagenesis system based on a modified Tn7 transposon, which promoted essentially random transposition into double-stranded DNA (Biery et al., 2000). This system was adapted to *H. pylori* and used to obtain the first genome saturating transposon library of *H. pylori* (Salama et al., 2004). Screening of these *H. pylori* mutant libraries has been very fruitful with the identification of
essential *H. pylori* genes (Bijlsma et al., 2000; Odenbreit et al., 1996a; Salama et al., 2004) and important *H. pylori* colonization factors (Baldwin et al., 2007; Kavermann et al., 2003), which consequently has improved our understanding of *H. pylori* biology and pathogenesis, and provided a number of potential new antimicrobial targets.

Genetic studies of *H. pylori* often entail the use of genetic elements that confer drug resistance to inactivate target genes or to introduce new DNA sequences by homologous recombination (Mobley et al., 2001). Although this strategy has been used in countless number of *H. pylori* studies this approach can be very limiting as transformants retain resistance determinants at targeted loci. Before the development of counterselection systems for *H. pylori*, studies that required changes at several loci in the same strain were difficult as only a few selectable resistance markers are available for *H. pylori* (Dailidiene et al., 2006). A counterselection system allows for the removal of resistance determinants that might affect downstream gene expression\(^1\), facilitates allelic exchange so that alleles with subtle differences (e.g., point mutation) or from different strains can be compared in a common genetic background, and permits recycling of the same resistance determinant for multiple use in the same strain. Several genes have been used to develop counterselection systems for genetic manipulation in bacteria (Blomfield et al., 1991; Fabret et al., 2002; Liu et al., 2008a; Marx, 2008; Russell and Dahlquist, 1989; Wang et al., 1994; Zhang et al., 2006) however, to date only two of these systems have been applied to *H. pylori* genetics. The first counterselection system developed for *H. pylori* used the *sacB* gene, encoding the enzyme levansucrase, (Steinmetz et al., 1983) which confers sensitivity to sucrose (Copass et al., 1997). The second counterselection system uses the *rpsL* (ribosomal protein S12) gene and is based on the dominance of wild-type streptomycin-sensitive (Str\(^s\)) alleles to resistance-conferring mutant alleles (Dailidiene et al., 2006; Lederberg, 1951). A novel, third counterselection system, developed specifically for *H. pylori* was reported only recently and uses the *rdxA* gene which confers susceptibility to metronidazole and must be used in *rdxA* null strains (Shaffer et al., 2011). These counterselection systems, though few, have served as very useful tools for the genetic manipulation of *H. pylori*, facilitating site directed mutagenesis or the in-frame deletion of many different target genes (Carpenter et al., 2009; Fischer et al., 2010; Genisset et al., 2006; Humbert et al., 2011; Schoep et al., 2010; Schweinitzer et al., 2008; Styer et al., 2010; Sycuro et al., 2012).

\(^1\) Non-polar cassettes, which are used to interrupt target genes without affecting the expression of downstream genes, have also been developed for use in *H. Pylori* (Skouloubris et al., 1998).
1.2.2 Complementation

Generally in genetic studies, to confirm the function attributed to a particular gene, a mutant strain in which the gene is disrupted must be complemented by introducing a wild-type copy of the gene at an alternate locus, and tested for the restoration of phenotypes that were lost in the mutant. Current methods used for gene complementation in *H. pylori* include shuttle plasmids and chromosomally based systems. Complementation at the *rdxA* (HP0954) locus (Smeets et al., 2000) is one of the most frequently used chromosomally based complementation systems in *H. pylori*, though several other systems have also been developed based on insertion into the intergenic region between HP0203 and HP0204, after *hpn* (HP1427) or within the *ureI* and *ureAB* loci (Bury-Monté et al., 2001; Eaton et al., 2002; Forsyth and Cover, 2000; Kang et al., 2004; Langford et al., 2006). However some of these systems can interrupt important chromosomal segments, making them unsuitable for *in vivo* studies, or can be susceptible to polar effects that may influence expression of other *H. pylori* genes (Langford et al., 2006).

An alternative to the chromosomally based systems are the shuttle plasmids, whose development has been another significant addition to the *H. pylori* genetic tool box. Shuttle plasmids are hybrids plasmids, comprised of a small *H. pylori* cryptic plasmid and the minimal essential genetic elements for *E. coli* plasmids, which are stable and can replicate autonomously in both hosts. To date, shuttle vectors based on three different cryptic plasmids have been reported. The first shuttle vector to be described was pBHP489K (Lee et al., 1997), however to date there is no literature evidence of its use by other research laboratories. Around the same time, a second shuttle vector system based on the *pHel1* cryptic plasmid was also reported (Heuermann and Haas, 1995, 1998). This system, shuttle vectors *pHel2* (*Cm*<sup>i</sup>) and *pHel3* (*Kan*<sup>i</sup>), has been used with great success by many different *H. pylori* research groups to not only complement inactivated chromosomal genes, but also to reintroduce genes modified by site-specific mutagenesis or to express foreign genes (Andrzejewska et al., 2006; Boneca et al., 2008; Fischer and Haas, 2004; Schweinitzer et al., 2008; Wunder et al., 2006). Unfortunately, for many *H. pylori* strains these shuttle plasmids transform at low frequencies or fail to transform altogether (Heuermann and Haas, 1998). Thus a third shuttle vector, pTM117, based on a cryptic plasmid found to be present in a large number of *H. pylori* strains was recently generated in an effort to develop a broadly
useful shuttle plasmid (Carpenter et al., 2007). However, the potential versatility for pTM117 remains unknown as its use has only been described in two H. pylori strains to date (Miles et al., 2010; Wen et al., 2007).

1.2.3 Conditional knockouts

Deletion mutants in combination with the use of animal models have been instrumental in the study of H. pylori, leading to significant advances in our understanding of H. pylori pathogenesis. However, the use of knockout mutants has several limitations. First, the inability to recover loss-of-function mutants can identify a target gene as essential for in vitro growth but the physiological role of the encoded protein may remain unknown using this strategy. Furthermore, it also remains unknown if a gene required for growth on agar plates, an artificial environment, is also required for growth in the pathogen’s natural environments or for survival under conditions that suppress replication but permit survival (e.g., the coccoid form of H. pylori) (Andersen and Rasmussen, 2009; Nilsson et al., 2002). Second, the use of knockout mutants does not allow for investigating whether a specific gene or set of genes encoding virulence determinants is necessary to maintain the infection state once it has been established or whether they are necessary for the entire infection cycle (Gandotra et al., 2007; Liu et al., 2008b). One approach that can overcome the limitations of constitutive loss-of-function mutants is the generation of conditional loss-of-function mutants in which transcription of the only functional copy of the target gene can be repressed. Such an approach would make it possible to study the physiological roles of essential genes and the temporal requirement of virulence determinants and other colonization factors during infection. This approach is of particular importance for the study of H. pylori pathogenesis as infection is persistent and clinical diseases develop after many years of chronic inflammation and epithelial damage (Atherton, 2006). The lack of suitable genetic systems to control gene expression in H. pylori has been a handicap in this area of research.

Conditional mutants have been generated in other bacteria using a variety of different systems, which encompass various regulatory mechanisms that are controlled by changes in temperature, pH or the availability of small molecules (Brautaset et al., 2009; Chou et al., 1995; Terpe, 2006; Valdez-Cruz et al., 2010). The first attempt at generating an inducible expression system for H. pylori used the endogenous H. pylori
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promoter of the iron-regulated pfr gene (Delany et al., 2002). However, *H. pylori* has only a few transcriptional regulators and they are central to mediating adaptive responses to changes in the environment, such as metal ion concentrations and external pH, by modulating the expression of an important number of genes (Danielli et al., 2010). Hence, the use of endogenous inducible promoters, that respond to iron or nickel concentrations, or genetic systems that are regulated by changes in pH or temperature would result in pleiotropic effects on *H. pylori* and complicate interpretation of conditional mutant phenotypes. Therefore, in the case of *H. pylori*, ideal gene regulation systems would be controlled by the presence of a small, non-toxic, cell-permeable molecule which cannot be synthesised by *H. pylori* and is not naturally available in its niche.

It has not been until very recently that the first such genetic tool for *H. pylori* has become available. Boneca and colleagues developed an inducible system based on the lacI-pTac system of *E. coli*, which permitted the generation of conditional *H. pylori* mutants to study the physiological function of essential genes (Boneca et al., 2008). This *H. pylori* adapted lac repressor system has been used with great success to study the physiological roles of essential penicillin-binding proteins and to elucidate the functional roles of trans-translation in *H. pylori* (Thibonnier et al., 2010; Thibonnier et al., 2008). Unfortunately, use of the lac repressor system is limited to *in vitro* studies only. This is because in order to regulate gene expression using the lac repressor system, it is necessary to use high concentrations of the inducer molecule (1 mM) making it impractical for studies involving animal models. Therefore, to study the temporal requirement of virulence determinants or to titrate gene products up or down during infection, it is necessary to develop an inducible expression system for *H. pylori* that can be regulated in animals.

Another inducible expression system that has great potential for generating conditional mutants in *H. pylori* is the tet repressor system of *E. coli* (Hillen and Berens, 1994). The tet repressor system uses the same regulatory principles as the lac repressor system to control gene expression, however the tet repressor’s affinity for its inducer is in the nanomolar range, clearly distinguishing it from other prokaryotic transcriptional regulators, such as the lac, pur, trp and tre repressors, which have micromolar affinities for their respective affectors (Scholz et al., 2003). The tet repressor system’s unique regulatory properties have resulted in its adaptation for use in many different organisms,
ranging from other prokaryotes to large animals (Berens and Hillen, 2003; Bertrand et al., 1983), and consequently has also been used to study bacterial pathogenesis in several infection models (Gandotra et al., 2007; Ji et al., 1999; Ji et al., 2001; Lathem et al., 2007).

Adaptation of the *tet* repressor system to *H. pylori* would generate a potentially very useful genetic tool for the study of *H. pylori* infection and its role in human health and disease.
1.3 The *tet* repressor system

The tetracycline dependent gene regulation system (*tet*-system) originated from a group of tetracycline resistance determinants (Hillen and Berens, 1994) that convey resistance to tetracycline in the form of active efflux of tetracycline from the cell.

Tetracycline (Tc) is an antibiotic that inhibits bacterial growth by binding to the bacterial ribosome, preventing the synthesis of new proteins. One mechanism whereby bacteria have developed resistance to the drug is to actively pump Tc out of the cell by way of a Tc efflux pump (Thaker et al., 2010). These efflux pump-based Tc resistance determinants are often located on plasmids and transposons, making them highly mobile and widely distributed in both gram-negative and gram-positive bacteria. Based on amino acid sequence similarity, there are 26 different classes of Tc efflux pumps, 18 of which can be classed into 6 groups (Thaker et al., 2010). Group-1 consists of Tc efflux pump classes Tet(A-E, G, H, J, Y, Z, 30, 31 and 33) and the resistance determinants that encode Group-1 Tc efflux pumps all share the same unique organization of structural and regulatory genes (Berens and Hillen, 2003; Hillen and Berens, 1994). The *tet*(B) class Tc resistant determinant found in the Tn10 transposon, carried on a conjugative plasmid R-100 in *E. coli* strain K-12 (Jorgensen and Reznikoff, 1979), has been extensively characterized to elucidate the regulation mechanism of Group-1 Tc resistant determinants (Hillen and Berens, 1994), and the components of the Tn10 Tc resistant determinant have been adapted to generate Tc-regulated conditional knockouts in both eukaryotic and prokaryotic systems.

1.3.1 Principle and origin

The Tn10-encoded Tc resistance determinant consists of two genes, *tetA* and *tetR*, which are orientated with divergent polarities and are transcribed from overlapping promoters. (Bertrand et al., 1983) (Figure 1-2 shows the genetic organization and mechanism of regulation of the Tn10-encoded Tc resistance determinant.)
Figure 1-2  Regulation of the Tn10-encoded Tc resistance determinant

The upper part of the figure shows a schematic drawing of the genetic organization and induction of tet genes, while the lower part depicts the processes occurring in the cytoplasmic membrane. The tet genes have divergent polarities and are transcribed by overlapping promoters. The tet repressor (orange circles), encoded by tetR, forms a homodimer and, in the absence of Tc, binds to two tandem tet operators, tetO₁ and tetO₂, preventing transcription of tetA and its own gene. Tc diffuses into the cell across the cytoplasmic membrane in the neutral form (bottom left side). Once in the cytoplasm, a divalent cation coordinates at O-11/O-12 of Tc to form the active [Tc·Me⁺] complex, which is the substrate of the proton-Tc antiporter, TetA, as depicted by its 12 alpha-helix membrane spanning structure on the bottom right. The same [Tc·Me⁺] complex, indicated by filled yellow triangles, is the molecular inducer of TetR. It binds to the repressor-operator complex and triggers a conformational change in TetR so that it can no longer bind the tet operators, resulting in rapid dissociation of TetR from the DNA. A burst of TetA and TetR expression follows and leads to a quick reduction in the cytoplasmic Tc concentration which, in turn, shuts down expression of both genes. (Adapted from (Hillen and Berens, 1994))
The resistance gene *tetA* encodes for a 12 transmembrane tetracycline/metal-proton (proton-[Tc-Me]+) antiporter which is located in the cytoplasmic membrane (Yamaguchi et al., 1990). The *tetR* gene encodes for the regulatory protein TetR, which is a Tc inducible repressor. TetR exists as a homodimer and, in the absence of Tc, binds to two tandemly orientated *tet* operators, *tetO₁* and *tetO₂*, shutting down transcription of *tetA* and of its own gene, *tetR*. (Hillen et al., 1983; Hillen et al., 1984) When Tc enters the cell it forms a [Tc-Me]+ complex and binds with high affinity to TetR (Takahashi et al., 1986). This induces a conformational change in TetR that abolishes its operator-binding specificity (Orth et al., 2000), resulting in dissociation of the repressor from the DNA, thus allowing DNA polymerase to access the promoter and a burst in expression of both TetA and TetR ensues. The induced TetA protein integrates into the cytoplasmic membrane where it exports [Tc-Me]+ in exchange for one proton (Yamaguchi et al., 1991). The removal of Tc from the cell results in free TetR which in turn shuts down expression of TetA and TetR.

Constitutive expression of TetA in the absence of Tc results in a severe disadvantage in competitive growth against other bacteria (Lee and Edlin, 1985; Nguyen et al., 1989) and overexpression of TetA is toxic to the cell (Eckert and Beck, 1989). Therefore, expression of TetA needs to be tightly repressed in the absence of Tc, yet it must be expressed before the cytoplasmic levels of Tc reach the micromolar levels necessary to inhibit protein synthesis. (Berens and Hillen, 2003; Hillen and Berens, 1994) This balance is achieved by the genetic organization of *tetA* and *tetR*, and by the ligand binding properties of TetR, making it a prime regulation system in terms of induction and repression.

The *tet* genes are differentially regulated so that repressor synthesis can occur before the resistance protein is expressed (Hillen and Berens, 1994). The two *tet* operators, situated within the overlapping, divergently arranged promoters of *tetA* and *tetR*, can be bound independently by TetR with extremely high affinity (Kamionka et al., 2004a). Occupation of *tetO₁* inhibits transcription of both *tetA* and *tetR* while occupation of *tetO₂* only represses the expression of *tetA* (Meier et al., 1988). The affinity of TetR for *tetO₂* is 2-fold greater than its affinity for *tetO₁* (Wissmann et al., 1986; Wray and Reznikoff, 1983). Therefore, upon a moderate decrease in operator bound TetR, a situation would be favoured in which *tetO₂* is occupied and *tetO₁* is not, permitting only the expression of TetR until both operators are occupied again and the expression of
both tet genes is shut down (Hillen and Berens, 1994), thereby preventing accidental expression of TetA in the absence of Tc. The binding constant of TetR for the [Tc·Me]+ complex is 10^3- to 10^5-fold higher than the affinity of the complex for the ribosome (Berens and Hillen, 2003), making TetR very sensitive to the presence of Tc in the cell. In addition, TetR has a very high affinity for tetO, which is reduced by nine orders of magnitude upon binding of two molecules of [Tc·Me]+, providing TetR with a very high ratio of specific over nonspecific DNA binding and enabling efficient binding to tetO (Lederer et al., 1996).

The evolutionary pressures on Tc resistance determinant gene regulation, to maintain efficient TetA repression without compromising sensitivity to induction so as to avoid the burden on fitness in the absence of Tc but still mediate high levels of resistance in its presence (Nguyen et al., 1989), have selected for a repressor that has a high affinity for both its DNA operator and its inducer, and displays very dramatic changes in operator affinity upon inducer binding. These qualities make the tet repressor an ideal tool for studies requiring controlled regulation of gene expression, and consequently components of the Tc resistance determinant gene regulatory system have been taken and adapted for use in many different research fields in biology. (Bertram and Hillen, 2008; Gossen and Bujard, 2002; Goverdhana et al., 2005; Stieger et al., 2009)

1.3.2 Use of the tet-system

Tc based regulation has been adapted to the study of other bacteria and to eukaryotic organisms. In early studies that adapted tet-regulation to new hosts, components of the Tc regulatory determinants remained unchanged (Gatz and Quail, 1988; Posfai et al., 1994; Skerra, 1994), however over the last two decades many modifications have been made both to the repressor protein and to the tet responsive promoter which have led to tet-systems that are more versatile and compatible with different host organisms (Berens and Hillen, 2003; Bertram and Hillen, 2008; Gossen and Bujard, 2002; Goverdhana et al., 2005; Nishijima et al., 2009; Schonig et al., 2010; Stieger et al., 2009).

1.3.2.1 Mutant TetR: changing its characteristics

Several X-ray crystallographic and mutagenesis studies on the tet repressor have identified the amino acid residues involved in, DNA operator recognition, inducer binding and dimer formation, and the combination of this crystallographic, biochemical
and genetic information has led to the elucidation of the mechanism through which binding of the inducer to TetR results in a dramatic decrease in TetR’s affinity for its DNA operator. (Baumeister et al., 1992; Hecht et al., 1993; Helbl et al., 1995; Helbl and Hillen, 1998; Helbl et al., 1998; Heuer and Hillen, 1988; Hinrichs et al., 1994; Kisker et al., 1995; Muller et al., 1995; Orth et al., 1998; Orth et al., 2000; Orth et al., 1999; Schnappinger et al., 1998; Scholz et al., 2000; Sizemore et al., 1990; Smith and Bertrand, 1988; Wissmann et al., 1991)

TetR forms a 46 kDa homodimer in which each monomer consists of 10 α-helices. The N-terminal DNA binding domain consists of helices α1 to α3, and contains a helix turn helix (HTH) motif that binds to the major groove of the DNA, recognizing the key palindromic residues in the tetO sequence. The C-terminal core domain consists of helices α5 to α10, and forms the two Tc binding pockets and the dimer interface with the second monomer (Figure 1-3 A and B). (Hinrichs et al., 1994; Kisker et al., 1995) The DNA binding domain is linked to the core domain via helix α4. Binding of the inducer to both Tc binding pockets initiates a conformational change through small displacements in helices α4, α6, α7 and α9, which leads to an increase in the distance between the two DNA binding domains of the TetR homodimer, with the result that the HTH motifs no longer fit into successive major grooves of the DNA helix and TetR dissociates from tetO. (Orth et al., 2000)

Extensive genetic and biochemical studies have led to the development of a repertoire of TetR variants with unique regulatory properties. TetR variants that have different operator specificities (Figure 1-4 A) have been generated by making amino acid substitutions of key residues in the DNA binding domain. (Baumeister et al., 1992; Helbl and Hillen, 1998; Helbl et al., 1998; Krueger et al., 2007) TetR variants with different inducer specificities have been generated through mutations to amino acid residues in the Tc binding pocket. Normally, TetR can only be induced by Tc and its analogs, like doxycycline (Dox) and anhydrotetracycline (ATc), that contain the 4-dimethylamino group (N(CH₃)₂) (Figure 1-4 B). However, the variant TetR H64K L131I S135L is exclusively induced by 4-de-dimethylamino-anhydrotetracycline and not by Tc, Dox or ATc, (Henssler et al., 2004) and the variant TetR N82A F86A is completely insensitive to tetracyclines and is instead induced by an altogether different class of molecule, a dodecapeptide known as Tip (Transcription inducing peptide) (Klotzsche et al., 2007).
Figure 1-3  TetR crystal structures

A. Structure of TetR-tetO complex. (Orth et al., 2000) TetR is depicted as a ribbon diagram with the α-helices labelled in one monomer. The DNA-binding domains are blue, helices that remain rigid in the core domain are yellow and helices undergoing conformational changes upon induction are green. The 15 bp operator fragment is represented by a red phosphate-ribose backbone and grey bases. This image is taken from Orth et al. (Orth et al., 2000).

B. Structure of TetR-[Tc-Me]+ complex. (Hinrichs et al., 1994) TetR is shown as a ribbon diagram with one monomer in grey and the other monomer is colour-coded: the DNA binding domain is magenta, the core domain is blue and the helix connecting the two domains is green. Tc is depicted as a space filling model in yellow, with two molecules bound to the two inducer binding pockets. This image is taken from Berens et al. (Berens and Hillen, 2003).
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Figure 1-4 Variations to tet-system components
Chapter 1: Literature review

Figure 1-4 Variations to tet-system components (adjacent page)
A. DNA sequences of tetO2 and three derived operator variants with single or double base-pair exchanges in the palindromic segment of tetO2.

B. Chemical structure of tetracycline (Tc), doxycycline (Dox), anhydrotetracycline (ATc) and 4-de-dimethylamino-anhydrotetracycline (4ddma-ATc).

C. Regulation principle of TetR (top) and revTetR (bottom). TetR is depicted by orange circles (DNA binding domain) and ovals (core domain) and revTetR is depicted by purple circles and ovals. The tet-regulated target gene is repressed or induced depending on the absence or presence of effector molecule (yellow triangle) bound to the repressor protein.

Several exhaustive mutagenesis studies have identified over one hundred repressor variants where two to five, or as few as one, amino acid substitutions has led to a reversal of function. These reverse tet repressors (revTetR), in contrast to TetR, require Tc for repression (Figure 1-4 C). (Gossen et al., 1995; Kamionka et al., 2004a; Scholz et al., 2004; Urlinger et al., 2000) Elegant studies have taken advantage of the different characteristics that can be generated by specific amino acid substitutions and the inability of TetR monomers from different Tc resistance determinant classes to form heterodimers (Schnappinger et al., 1998). They have demonstrated, in both bacterial and mammalian cell culture, that TetRs from different classes, with altered operator and inducer specificities, can be used together to develop a double tet-repressor system. This involved using different tetracycline analogs, to specifically regulate one TetR protein variant over another, so as to selectivity regulate the expression of two different genes within the same cell (Kamionka et al., 2004b; Krueger et al., 2004).

1.3.2.2 Tet-regulation in prokaryotes

Tet-regulated expression systems have been developed from Tc resistant determinants (discussed in section 1.3.1.) and used in 20 different bacterial species to date (Table 1-1). The main obstacle faced in translating tet-regulation to other bacterial species has been the functional optimization of the tet-responsive promoter for the host bacterium.

The first systems developed to regulate gene expression in E. coli used the unmodified Tn10 tetA promoter, Ptet, (de la Torre et al., 1984; Posfai et al., 1994; Skerra, 1994). Repression of target gene expression by TetR was very tight however tet-induced expression from Ptet was only moderate. The system was improved upon when a synthetic tet-sensitive promoter, P_{LtetO-1}, was developed by replacing the phage lambda
CI repressor binding sites in the phage lambda P_L promoter with tetO sequences (Lutz and Bujard, 1997). This synthetic system could establish large regulatory windows of up to 5000-fold induction of gene expression. P_{tet} and P_{LtetO-1} are the tet-responsive promoters that have been routinely used in E. coli and other γ-proteobacteria such as Salmonella enterica serovar Typhi (Qian and Pan, 2002), Yersinia pestis (Lathem et al., 2007) and Vibrio cholerae (Hsiao et al., 2006), and have recently become commercially available from Clontech. The Tn10-based tet-promoters are inactive in Bacillus subtilis and so TetR mediated regulation in gram-positive bacteria was achieved by addressing the different promoter requirements of these organisms. The promoter driving TetR expression was improved by changing the -35 and Shine-Dalgarno sequences and a tet-responsive promoter, P_{xyl/tet}, was made by modifying the strong B. subtilis xylA promoter with one or two tet operators (Geissendorfer and Hillen, 1990). This gram-positive adapted system has subsequently been used to study gene function not only in pathogenic Staphylococci and Streptococci, but also in mollicutes, spirochaetes and the recently characterized β-proteobacteria Laribacter hongkongensis (Table 1-1).

Tet-regulation has also been successfully adapted for use in Actinobacteria. Conditional expression systems were available for Mycobacteria, however due to their inefficiency there was still a need for a system that offered both tight repression and regulation in vivo (Blokpoel et al., 2005; Ehrt et al., 2005). Consequently tet-regulation was independently developed in Mycobacteria by four different research groups. Ehrt et al. constructed a Tn10-derived tet-expression system by constitutively expressing tetR from a Mycobacterium smegmatis promoter and by replacing sequences of the M. smegmatis rpsA promoter with tetO sites to generate a mycobacterial tet-responsive promoter, P_{myc1tetO} (Ehrt et al., 2005). Blokpoel et al. imported the tetZ Tc resistance determinant from the more closely related Corynebacterium glutamicum (Blokpoel et al., 2005), Carrol et al. used the P_{xyl/tet} promoter system developed in B. subtilis (Carroll et al., 2005), while Hernandez-Abanto et al. introduced tet-regulation in M. smegmatis by importing the Tn10-based P_{tcp1tetO} system developed for Streptomyces (Hernandez-Abanto et al., 2006; Rodriguez-Garcia et al., 2005).

Despite originating from bacteria, the tet-system has only been adapted and improved for use in other bacteria in the last decade. Interestingly, much more significant headway has been made in adapting the tet-system for gene regulation in eukaryotic systems.
<table>
<thead>
<tr>
<th>Species</th>
<th>Class</th>
<th>Application</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
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<td></td>
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<tr>
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<td><em>In vitro</em></td>
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<td><em>In vitro and in vivo</em></td>
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</table>
Chapter 1: Literature review

1.3.2.3 Tet-regulation in eukaryotes

Tc controlled gene expression in eukaryotes was achieved by taking the essential regulation elements of the Tn10 Tc resistance determinant and modifying them to meet eukaryotic requirements. Initially, unmodified TetR was transcribed from a strong constitutive eukaryote promoter and multiple tet operators were positioned in proximity to the TATA-element and transcriptional start site of the target gene. Unmodified TetR served as an efficient transcriptional repressor in plants and lower eukaryotes, but has not always worked efficiently in mammalian cells (Berens and Hillen, 2003).

Seminal work by Glossen and Bujard facilitated the complete transformation of the prokaryotic Tn10-derived tet-system into a genetic switch for controlling gene expression in potentially all eukaryotes (Gossen and Bujard, 1992). They fused the herpes simplex virus transcription activator (VP16) to the C-terminus of TetR, converting it into a tet-controlled transcriptional activator, tTA, and they generated a synthetic tet-responsive promoter (P_{tet}) by fusing seven repeats of the tetO2 sequence to a minimal human cytomegalovirus (CMV) immediate early promoter (for clarity, the eukaryote P_{tet} promoter will be referred to as eP_{tet}). In the presence of Dox, transcription from eP_{tet} is silent, however upon removal of Dox, tTA binds to the tetO sequences and the C-terminal VP16 activation domain recruits components of the RNA polymerase II transcription initiation complex to initiate transcription of the target gene. Thus, in Glossen and Bujard’s tet-system, constitutively expressed tTA initiates and drives transcription of the target gene, while in the presence of Dox, tTA is unable to bind eP_{tet} and eP_{tet}-controlled transcription is turned off (Tet-Off). The revTetR equivalent of tTA, reverse tTA (rtTA), was later obtained through four point mutations in the TetR domain of tTA, resulting in the development of eukaryotes tet-system where Dox is necessary for rtTA to bind to eP_{tet} and turn eP_{tet}-controlled transcription on (Tet-On). (Gossen et al., 1995)

The eukaryote tet-system has been further improved by targeting tTA to the nucleus, optimizing its codon usage (Sprengel and Hasan, 2007), exchanging the VP16 activation domain with other activation or repression domains, and by using alternative promoters to drive constitutive expression of tTA (Berens and Hillen, 2003). Consequently, tet-regulation has to date been used in cultured cells from mammals, plants, amphibians and insects, and with the use of tissue specific promoters the tet-system has been used to regulate gene expression in whole organisms, including plants,
flies, mice, rats (Berens and Hillen, 2003; Gossen and Bujard, 2002; Nishijima et al., 2009) and recently even in dogs (Kim et al., 2011). Extensive transgenic work in mice, using tet-controlled gene expression in both a temporal and tissue specific manner, has provided fundamental insights into various biological processes such as development, disease and behaviour (Schonig et al., 2010; Sprengel and Hasan, 2007).

The successful adaptation of the tet-system to the study of gene expression in whole organisms established that tet-regulation could also be applied to study bacterial pathogenesis in animal infection models and is likely to have rekindled a renaissance of the tet-system in bacteria.

1.3.2.4 Use of tet-regulation to study bacterial pathogenesis

The significant advances made using the tet-system in eukaryotes demonstrated that tet dependent gene expression was possible in living animals and that gene expression could be selectively regulated in all the major organs and at the extremities (Hasan et al., 2001; Kistner et al., 1996). This meant that tet-regulation in prokaryotes was no longer limited to protein expression and the study of essential genes, but importantly, could also be applied to the study of bacterial pathogenesis in animal infection models. The tet-system is an ideal tool for this purpose as the regulatory concentrations of tetracyclines are sub-inhibitory and they can reach the site of infection to act on bacterial gene expression.

Combining tet-regulation with animal infection models can elucidate the temporal requirements of different genes in an infection cycle, led to a better understanding of the infection and also identify and validate antimicrobial targets (Gandotra et al., 2007). To date, tet-regulation has been used in animal infection models for *M. tuberculosis* (Gandotra et al., 2007), *Y. pestis* (Lathem et al., 2007) and *S. aureus* (Ji et al., 1999; Ji et al., 2001). Using the tet-system to silence genes encoding the *M. tuberculosis* core proteasome at different time points after mouse infection, it was demonstrated not only that the *M. tuberculosis* proteasome is required for optimal growth in vivo, but that it is also essential during the chronic phase of the infection, when the pathogen is replicating slowly or not at all (Gandotra et al., 2007). The role of the *Y. pestis* encoded plasminogen activator, Pla, in the development of primary pneumonic plague in a mouse infection model has also been investigated (Lathem et al., 2007). Using tet-regulation to manipulate the temporal expression of Pla, it was demonstrated that Pla
allows *Y. pestis* to replicate rapidly in the airways and cause lethal fulminant pneumonia. However, when not expressed, inflammation is aborted and lung repair is activated. Furthermore this study identified Pla as a therapeutic target to extend the period for effective antibiotic treatment, as inhibition of Pla 36 h after infection prolonged survival of animals from 3.1 to 4.6 days. Other studies have combined *tet*-regulation with antisense technology in animal models for *S. aureus* infection (Ji et al., 1999; Ji et al., 2001). In this arrangement, induction of *tet*-regulated antisense RNA resulted in inhibition of target gene expression. Using this system, 150 *S. aureus* genes were identified as either essential or critical to growth (Ji et al., 2001). Furthermore, this study demonstrated that the conditional phenotypes could be titrated, both *in vitro* and *in vivo*, in an ATc dose-dependent manner. This tool will be extremely useful for determining to what extent *S. aureus* antimicrobial targets need to be inhibited to achieve effective clinical outcomes.

In conclusion, *tet*-regulation would be an ideal genetic tool to study *H. pylori* infection. Repression by TetR is efficient, expression of the gene of interest is conditional and dose-responsive, effector concentrations are sub-inhibitory and can regulate expression in an animal host, thus permitting the study of *H. pylori* in the context of an intact gastric environment. Initial *H. pylori* infection results in acute active gastritis which develops into chronic active gastritis that lasts for the individual’s lifetime if left untreated. How is this infection maintained for so many decades, by what mechanism does *H. pylori* polarize the adaptive immune response and in what way does *H. pylori* control the disease outcomes in asymptomatic (~90%), peptic ulcer (~10%) or gastric cancer (~1%) patients, are only a few of the important questions that *tet*-regulation could address.
1.4 Project aims

As discussed above there are a limited number of genetic tools available to study *H. pylori* pathogenesis and persistence. In particular, genetic tools that can be used in animal models to study the functional role and temporal requirements of *H. pylori* virulence determinants during infection are lacking. The main aim of this study is to adapt the tet repressor system to *H. pylori* and demonstrate that it can be used to generate conditional *H. pylori* mutants where gene expression can be controlled both *in vitro* and *in vivo* during the course of infection.

Due to the nature of the main study presented in this thesis, and the intellectual property surrounding it, a second minor project was also undertaken to permit free presentation at scientific conferences. This work involved the characterization of the dif/XerH system in *H. pylori* which facilitated the adaptation of Xer-cision as a new genetic tool for the expedited generation of markerless *H. pylori* mutants, and is presented as two manuscripts in Chapter 7.
## 2 General materials and methods

### 2.1 General materials

#### 2.1.1 Reagents

##### 2.1.1.1 Molecular biology reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampule saline</td>
<td>Pfizer</td>
</tr>
<tr>
<td>Ampule water</td>
<td>Pfizer</td>
</tr>
<tr>
<td><strong>Antibodies</strong></td>
<td></td>
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<tr>
<td><em>Antibodies for Elisa</em></td>
<td></td>
</tr>
<tr>
<td>• Goat Anti-mouse IgA (α chain specific)</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Alkaline Phosphatase conjugate</td>
<td></td>
</tr>
<tr>
<td>• Goat Anti-rabbit IgG (whole molecule)</td>
<td>Sigma-Aldrich, USA</td>
</tr>
<tr>
<td>Alkaline Phosphatase conjugate</td>
<td></td>
</tr>
<tr>
<td>• Rabbit polyclonal Anti-<em>Helicobacter pylori</em></td>
<td>Dako, Australia</td>
</tr>
<tr>
<td>• Sheep Anti-mouse IgG (whole molecule) F(ab)(_2) fragment Alkaline Phosphatase conjugate</td>
<td>Sigma-Aldrich, USA</td>
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<td><strong>Antibodies for Flow cytometry</strong></td>
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<tr>
<td>• APC labelled Anti-mouse CD19 (ID3)</td>
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</tr>
<tr>
<td>• APC labelled Rat Anti-mouse CD11b (M1/70)</td>
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</tr>
<tr>
<td>• APC-Cy(_{TM}) labelled Rat Anti-mouse ly-6G clone 1A8</td>
<td>BD Pharmingen, USA</td>
</tr>
<tr>
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<td>• PE Cy(_{TM}) labelled Rat Anti-mouse CD4 (RM4-5)</td>
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<tr>
<td>• Per CP-Cy(_{TM})5.5 labelled Rat Anti-mouse ly-6C</td>
<td>BD Pharmingen, USA</td>
</tr>
</tbody>
</table>

### Antibodies for Fluorescence Microscopy
- Alexa Fluor® 488 labelled Goat Anti-chicken IgG
- Chicken Anti-GFP
- DyLight 549 labelled Goat Anti-rabbit IgG
- Rabbit monoclonal Anti-*Helicobacter*

### Primary antibodies for Immunoblotting
- Mouse monoclonal Anti-HA (isolated from culture of CM1-1 hybridoma cell line WC00009)
- Mouse monoclonal Anti-UreB
- Rabbit polyclonal Anti-GFP - sera
- Rabbit polyclonal Anti-urease - sera
- Rabbit polyclonal IgG Anti-TetR

### Secondary antibodies for Immunoblotting
- Mouse Anti-rabbit IgG HRP
- Rabbit Anti-mouse IgG HRP

### DNA extraction and purification kits
All kits used in this study were used as directed in the instruction manual supplied by the manufacturer.
- PureLink™ Genomic DNA Kit
- PureLink™ PCR Purification Kit
- PureLink™ Quick Gel Extraction
- PureLink™ Quick Plasmid Miniprep Kit

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### Other Reagents
- BD Microtainer® Tubes SST™
- BD™ Stabilizing Fixative
- Carbon Fuchsin diluted stain
- Carnoy’s fixative
- Crystal violet 0.5% aqueous stain solution
- Dithiothreitol (DTT)
- DNA extraction and purification kits
- Forane (Isoflurane)
- Glutaraldehyde, 25% solution
- Gram’s Iodine stain
- Hoechst 333421
- LIVE/DEAD® Fixable Aqua Dead Cell Stain
- Paraformaldehyde

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PCR reagents
All DNA polymerases were used with the recommended buffer provided by the manufacturer.

- Accuprime\textsuperscript{TM} Pfx SuperMix\newline Invitrogen Life Technologies, USA
- BigDye\textsuperscript{®} Terminator ready reaction mix v3.1\newline ABI, USA
- Deoxyribonucleoside triphosphates (dNTPs)\newline Invitrogen Life Technologies, USA
- MyTaq\textsuperscript{TM} DNA polymerase\newline Bioline Meridian Life Science
- Pwo Superyield DNA polymerase\newline Roche Applied Sciences, Germany
- Taq DNA polymerase\newline Roche Applied Sciences, Germany

Phenol Red\newline Sigma-Aldrich, USA
PMSF (100 mM solution)\newline Sigma-Aldrich, USA

Restriction endonucleases
All restriction endonucleases were used with the recommended buffer provided by the manufacturer.

- BamHI\newline New England BioLabs, USA
- BglII\newline New England BioLabs, USA
- Clal\newline New England BioLabs, USA
- DpnI\newline New England BioLabs, USA
- EcoRI\newline New England BioLabs, USA
- HindIII\newline New England BioLabs, USA
- NcoI\newline New England BioLabs, USA
- NdeI\newline New England BioLabs, USA
- SacII\newline New England BioLabs, USA
- SalI\newline New England BioLabs, USA
- SfiI\newline New England BioLabs, USA
- XbaI\newline New England BioLabs, USA
- XhoI\newline New England BioLabs, USA

Rubidium Chloride (RbCl)\newline Sigma-Aldrich, USA
O. C. T. Compound\newline Tissue Tek®, ProSciTech, Australia

Other DNA modifying enzymes
All enzymes were used with the recommended buffer provided by the manufacturer.

- Calf intestinal phosphatase (CIP)\newline New England BioLabs, USA
- T4 DNA ligase\newline Roche Applied Science, Germany
- T4 DNA polymerase\newline New England BioLabs, USA

SDS Page and Western blot reagents

- 30% Acrylamide/Bis Solution\newline Bio-rad Laboratories
- Ammonium persulfate\newline Bio-rad Laboratories
- Bovine Serum Albumin (BSA)\newline Sigma-Aldrich Corporation, USA
- Bradford reagent\newline BioRad
- Chemiluminescent Peroxidase Substrate-3\newline Sigma-Aldrich Corporation, USA
- Coomassie brilliant R450\newline Bio-rad Laboratories
## Chapter 2: General materials and methods

- **Pre-stained** Protein Marker: Fermentas Life Sciences, USA
- **Polyoxyethylene sorbitan monolaurate** (Tween-20): Sigma-Aldrich Corporation, USA
- **PVDF** membrane: Sigma-Aldrich Corporation, USA
- **TEMED**: Bio-rad Laboratories
- **SIGMAFAST™** p-Nitrophenol phosphate tablets (pNPP): Sigma-Aldrich, USA

### Stains and DNA ladders

- DNA marker: 1 kb ladder: New England Biolabs, USA
- DNA marker: 1 kb ladder: Roche Applied Sciences, Germany
- DNA marker: λ DNA-HindIII digest: New England Biolabs, USA
- SYBR™ Green: Invitrogen Life Technologies, USA
- SYBR™ Safe: Invitrogen Life Technologies, USA

### TA cloning kits/plasmids

- pGEM®-T Easy Vector systems: Promega
- Urea: Merck, USA
- Urease from *Canavalia ensiformis* (Jack bean): Sigma-Aldrich Corporation, USA
- X-gal: Fisher Science, Australia

#### 2.1.1.2 Culture reagents

<table>
<thead>
<tr>
<th>Reagent</th>
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<td>Bacto-tryptone</td>
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<td>BHI with 20% glycerol</td>
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<td>Blood agar plates</td>
<td>PathWest Laboratory Medicine, Australia</td>
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<td>Brain Heart Infusion broth (BHI) base</td>
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<td>Brucella broth</td>
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<td>Defibrinated horse blood</td>
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<td>Yeast Extract</td>
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</table>
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2.1.1.3 Oligonucleotide primers
Oligonucleotides were designed using the Vector NTI® Advance 10 and 11 software (Invitrogen Life Technologies, USA). Oligonucleotides were synthesized by either Geneworks (Australia), Invitrogen (New Zealand), or Ocimum Biosolutions (India) and cartridge purified. All primers used in this study were reconstituted in sterile H₂O and stored at -20 °C (see section 2.2.2.3). The primers used in this study are listed in Chapter 3 (Table 3-3).

2.1.1.4 Chemical reagents
All chemicals were purchased from Sigma-Aldrich, Australia and were used and stored according to the manufacturer’s instructions.

Table 2-3 Source of specific chemical reagents and materials used for thin layer chromatography

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<td>TLC plates</td>
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</table>

2.1.1.5 Plasticware
All general disposable plasticware such as pipettes, 15 and 50 ml tubes, PCR tubes and petri dishes, were purchased from several suppliers including, Falcon BD, Corning and Starsted.

Table 2-4 Source of specific plasticware used in this study

<table>
<thead>
<tr>
<th>Consumable</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well plate, clear, round bottom</td>
<td>Falcon, BD</td>
</tr>
<tr>
<td>96 well plate, black</td>
<td>Nunc</td>
</tr>
<tr>
<td>UV transparent 96 well plate</td>
<td>Corning</td>
</tr>
<tr>
<td>MaxiSorp® 96 well ELISA plate</td>
<td>Nunc</td>
</tr>
<tr>
<td>75 cm vented tissue culture flask</td>
<td>Corning</td>
</tr>
</tbody>
</table>
2.1.2 Plasmids and bacterial strains

2.1.2.1 Plasmids and synthetic constructs

pBlu-SK-alt
pBlu-SK-alt was generated from pBluescript SK (-) by Ondek Pty. Ltd. (Carola Schwan). The plasmid pBluescript SK (-) (Invitrogen, Australia) was treated with XhoI and SalI restriction endonucleases and re-ligated to give pBlu-SK-alt. This treatment destroyed the unique XhoI and SalI restriction sites in the multiple cloning site.

pHSG576
pHSG576 is a low copy plasmid and was used as a cloning vector for genetic modification of the *H. pylori* strains. This vector was obtained from Dr. Charlene Kahler (Perth, Australia).

pGEM®-T Easy vector
pGEM®-T Easy vector was used for TA cloning of PCR products. This vector was purchased from Promega, Australia.

pSK485
Several DNA constructs in this study were ordered from and synthesized by GeneART®, Invitrogen. These synthetic sequences were received from the manufacturer as inserts cloned into the pSK485 vector backbone, which harbours a Col E1 origin of replication and *bla*, encoding Amp'.

pUC18K
pUC18K was used as a source for the *alpha* gene, encoding a non-polar Kanamycin resistance cassette.

pWH1925 BD and pWH1925 r2
Served as DNA template for the amplification and cloning of sequences encoding TetR and revTetR respectively. These plasmids were obtained from Prof. Wolfgang Hillen (Erlangen, Germany).
Chapter 2: General materials and methods

**pONDG**
Plasmid pONDG was obtained from Dr. Ji Kevin Li. This was used as a template sequence for amplifying \textit{gfp}mut2.

**pMdaB and pMdaB-RCAT**
pMdaB and pMdaB-RCAT are derivatives of the pBlu-SK-alt plasmid, and were made by Ondek Pty. Ltd. (Carola Schwan). Plasmid pMdaB contains a 2 kb region of \textit{H. pylori} genomic DNA, the last 500 bp of HP0629 to the first 836 bp of HP0631 inclusively. Inserted between the HP0630 and HP0631 coding regions is a small multiple cloning site. pMdaB-RCAT is a derivative of pMdaB that contains the counterselective cassette, \textit{rpsL-cat}, (Dailidiene et al., 2006) cloned into the \textit{BamHI} restriction site.

**pENT-RC**
pENT-RC is a derivative of pEntranceposon (Tet\textsuperscript{r}) from Finnzymes (F-767) where the Tetracycline resistance marker has been replaced with a \textit{rpsL-cat} counterselectable marker (Dailidiene et al., 2006). This plasmid was made by Ondek Pty. Ltd. (Carola Schwan) and was the source for the \textit{rpsL-cat} counterselection cassette in this study.

\textbf{2.1.2.2 \textit{E. coli} strains}

\textit{E. coli} strain DH5\textalpha{} was used as a host strain for cloning and genetic manipulations. This strain was obtained from the -80 °C stocks in the Discipline of Microbiology and Immunology at the University of Western Australia. \textit{E. coli} strains used for the specific aims are listed in Table 3.2. All strains were stored at -80 °C in the \textit{H. pylori} Research Laboratory and at Ondek Pty. Ltd., in the Discipline of Microbiology and Immunology at the University of Western Australia.

\textbf{2.1.2.3 \textit{H. pylori} strains}
The source and description of \textit{H. pylori} strains used in this study are listed as follows. The \textit{H. pylori} strains used for the specific aims are listed in each chapter. All strains were stored at -80 °C in the \textit{H. pylori} Research Laboratory and at Ondek Pty. Ltd., in the Discipline of Microbiology and Immunology at the University of Western Australia.
H. pylori 26695
Strain 26695 was isolated from a patient in the United Kingdom with gastritis and was the first completely sequenced H. pylori strain (Tomb et al., 1997). This strain was obtained from the American Type Culture Collection (ATCC).

H. pylori X47-2AL
Strain X47-2AL (X47) was originally isolated from a domestic cat (Handt et al., 1995), and was adapted to mice by sequential in vivo passages (Kleanthous et al., 2001). This strain was obtained from Prof. Douglas E. Berg (Washington University, St. Louis, USA).

2.1.3 Culture media
All media was sterilised by autoclaving at 121 °C for 15 min. Media was cooled to 50 °C before the addition of antibiotics and supplements. Approximately 20 ml of molten agar was used per petri dishes and allowed to set for 30 min at room temperature before storage in a sealed bag at 4 °C. All broth was stored at 4 °C and equilibrated to 37 °C before use.

2.1.3.1 Culture media to grow E. coli
SOC Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-tryptone</td>
<td>20 g/l</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g/l</td>
</tr>
<tr>
<td>5 M NaCl</td>
<td>2 ml/l</td>
</tr>
<tr>
<td>1 M KCl</td>
<td>2.5 ml/l</td>
</tr>
<tr>
<td>1 M MgCl₂</td>
<td>10 ml/l</td>
</tr>
<tr>
<td>1 M MgSO₄</td>
<td>10 ml/l</td>
</tr>
<tr>
<td>1 M glucose</td>
<td>20 ml/l</td>
</tr>
</tbody>
</table>

Luria-Bertani (LB) Agar

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-tryptone</td>
<td>10 g/l</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g/l</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g/l</td>
</tr>
<tr>
<td>Bacto-agar</td>
<td>15 g/l</td>
</tr>
</tbody>
</table>
LB Broth

- Bacto-tryptone: 10 g/l
- Yeast extract: 5 g/l
- NaCl: 10 g/l

2.1.3.2 Culture media to grow *H. pylori*

**Brain Heart Infusion broth (BHI)**

- BHI: 37 g/l
- NCS: 10% (v/v)

**Brucella broth**

- BBL Brucella: 28 g/l
- NCS: 10% (v/v)

**Columbia blood agar (CBA) plates**

- Columbia agar: 39 g/l
- Horse blood: 5% (v/v)

**Heart Infusion Broth (HI)**

- HI: 39 g/l
- NCS: 10% (v/v)

2.1.3.3 Antibiotics

To select for recombinant *E. coli* and *H. pylori*, growth media were supplemented with antibiotics to the concentrations shown in Table 2-5. All antibiotics were purchased from Sigma-Aldrich in powdered form. Stock solutions were made as listed and stored at -20 °C.

**Dent selective supplement**

Dent selective supplement was supplied in a lyophilized form by Oxoid, England and stored at 4 °C. The content of the vial was dissolved in 2 ml ddH₂O and was added to 475 ml of sterile Columbia Blood agar base cooled to 50-55 °C with 25 ml horse blood. This was mixed well and poured into sterile petri dishes.
Table 2-5  Stock and working concentrations of antibiotic solutions used in this study

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Stock solution concentration</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>100 mg/ml in H2O</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>10 mg/ml in Ethanol</td>
<td>25 μg/ml</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>10 mg/ml in H2O</td>
<td>10 μg/ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>100 mg/ml in H2O</td>
<td>-</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>5 mg/ml in DMSO</td>
<td>-</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>100 mg/ml in H2O</td>
<td>-</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>10 mg/ml in 0.1M NaOH</td>
<td>-</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>10 mg/ml in H2O</td>
<td>-</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>5 mg/ml in DMSO</td>
<td>-</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>250 mg/ml in H2O</td>
<td>-</td>
</tr>
<tr>
<td>Cefsulodin</td>
<td>5 mg/ml in H2O</td>
<td>-</td>
</tr>
</tbody>
</table>

1 All antibiotics were stored at -20 °C, except Polymyxin B.
2 Antibiotics used in selective plates for isolation of H. pylori from mouse stomach tissue.
3 Polymyxin B stock was stored at 4 °C.

2.1.4  Buffers and Solutions

Unless otherwise stated, all buffers and solutions were prepared in deionised distilled water (ddH2O), and were sterilized by autoclaving at 121 °C for 15 min. For solutions that were sterilized by filtration, a 0.22 μm filter was used. All pH adjustments were made using either 1 M HCl or 1 M NaOH where required. The solutions were prepared either according to manufacturer’s instructions or according to methods described in Molecular Cloning: A laboratory manual (Sambrook and Russel, 2001).

2.1.4.1  Stock solutions

Deoxyribonucleoside triphosphates (dNTPs)

The dNTP set (including dATP, dCTP, dGTP, dTTP, 100 mM each) was purchased from Invitrogen. The contents of each vial (200 μl) were mixed together and 9.2 ml of ddH2O was added to make 10 ml of a 2 mM stock of dNTPs. The dNTPs mix was stored in 25 μl aliquots at -20 °C.
EDTA (0.5 M, pH 8.0)
Disodium ethylenediaminetetraacetic acid dihydrate (186.1 g) was added to 800 ml of 
H₂O with vigorous stirring on a magnetic stirrer. The pH of the solution was adjusted to 
8.0 with 1 M NaOH. The solution was sterilized by autoclaving.

Glucose (1 M)
A 1 M solution of glucose was prepared by dissolving 90.08 g of glucose in 450 ml of 
H₂O. Volume was adjusted to 500 ml with H₂O and sterilized by filtration and stored at 
room temperature.

HCl (1 M)
A 1.72 ml aliquot of hydrochloric acid (10 M) was added to 16 ml H₂O and the total 
volume was made up to 20 ml. The solution was sterilized by autoclaving and stored at 
room temperature.

KCl (1 M)
Potassium chloride (74.5 g) was dissolved in 800 ml of H₂O. The volume was adjusted 
to one litre with H₂O and stored at room temperature.

MgCl₂ (1 M)
Magnesium chloride (9.52 g) was dissolved in 80 ml of H₂O. The volume was adjusted 
to 100 ml with H₂O and sterilized by filtration. The solution was stored at 4 °C.

MgSO₄ (1 M)
Magnesium sulphate (12.36 g) was dissolved in 80 ml of H₂O. The volume was 
adjusted to 100 ml with H₂O and sterilized by filtration. The solution was stored at 4 °C.

NaCl (5 M)
A 5 M solution of sodium chloride was prepared by dissolving 292.2 g of NaCl in 
800 ml of H₂O. The volume was adjusted to one litre with H₂O and stored at room 
temperature.
**NaOH (10 M)**
Sodium hydroxide pellets (400 g) were slowly added into 800 ml of H₂O with continual stirring. When the pellets were dissolved completely, the volume was adjusted to one litre with H₂O. The solution was stored in a plastic container at room temperature.

**NaOAc (3 M, pH 5.2)**
Sodium acetate trihydrate (40.8 g) was dissolved in 80 ml of H₂O. Glacial acetic acid was used to adjust the pH to 5.2. The volume was adjusted to one litre with H₂O and sterilized by autoclaving.

**SDS (10% w/v)**
SDS is also called sodium dodecylsulphate. Electrophoresis-grade SDS (100 g) was dissolved in 900 ml of H₂O. The solution was heated to 68 °C and stirred with a magnetic stirrer. The volume was adjusted to one litre with H₂O and stored at room temperature.

**SYBR™ Green solution (100×)**
SYBR™ Green was purchased from Invitrogen as 10,000× concentrated stock solution and diluted with DMSO to give a 1:100 solution. The SYBR™ Green solution was stored in the dark at -20 °C.

**X-gal solution (2% w/v)**
X-gal is 5-bromo-4-chloro-3-indolyl-β-D-galactoside. A 2% (w/v) stock solution was prepared by dissolving X-gal in DMSO at 20 mg/ml. The tube containing the solution was wrapped in aluminium foil to prevent damage by light and stored at -20 °C.

### 2.1.4.2 General buffers

**Blocking buffer**
Blocking buffer was prepared by dissolving 2 g of bovine serum albumin (BSA) in PBST to a final volume of 50 ml. The solution was prepared just before use.
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**Phosphate-buffered Saline (PBS) (10× and 1×)**
One litre of 10× PBS buffer was prepared by dissolving the following reagents in 800 ml of H$_2$O:

- NaCl: 80 g
- KCl: 2 g
- Na$_2$HPO$_4$: 14.4 g
- KH$_2$PO$_4$: 2.4 g

The pH was adjusted to 7.4 and the total volume was made up to 1 litre. The 1× PBS working solution was prepared by diluting 100 ml of 10× PBS (stock solution) in one litre of H$_2$O. The PBS solutions were sterilized by autoclaving and stored at room temperature.

**PBST**
1× PBS containing 0.1% Tween-20 (v/v).

**TE buffer (pH 7.6)**
Tris-HCl (1.57 g) and 0.37 g of EDTA were dissolved in 990 ml H$_2$O. The pH was adjusted to 7.6 and the total volume was made up to one litre. The TE buffer was sterilized by autoclaving and stored at room temperature.

**Tris (1 M)**
One litre of 1 M Tris was prepared by dissolving 121.1 g Tris-base in 800 ml of H$_2$O. The pH was adjusted to the desired value by the addition of concentrated HCl and the total volume was made up to one litre. The solution was sterilized by autoclaving.

**Tris (1.5 M, pH 8.8)**
One litre of 1.5 M Tris was prepared by dissolving 181.7 g Tris-base in 800 ml of H$_2$O. The pH was adjusted to 8.8 by the addition of concentrated NaOH and the total volume was made up to one litre. The solution was sterilized by autoclaving.

**Tris (0.5 M, pH 6.8)**
One litre of 0.5 M Tris was prepared by dissolving 60.5 g Tris-base in 800 ml of H$_2$O. The pH was adjusted to 6.8 by the addition of concentrated HCl and the total volume was made up to one litre. The solution was sterilized by autoclaving.
2.1.4.3 Electrophoresis buffers

**Electrotransfer buffer (10× and 1×)**

One litre of 10× electrotransfer buffer was made by dissolving the following reagents in 900 ml of H$_2$O:

- Tris-base: 3.03 g
- Glycine: 14.4 g

The volume was adjusted to one litre with H$_2$O. The 1× electrotransfer buffer working solution was prepared by diluting 100 ml of 10× electrotransfer buffer (stock solution) in 800 ml of H$_2$O and added to 200 ml of absolute methanol. The working solution was prepared and chilled just before use.

**SDS-PAGE running buffer (10× and 1×)**

One litre of 10× stock Tris-glycine SDS-PAGE running buffer was prepared by dissolving the following reagents in 900 ml of H$_2$O:

- Tris-base: 30.3 g
- Glycine: 144 g
- SDS: 10 g

Total volume was made up to one litre with H$_2$O. The solution was prepared as a concentrate and diluted to 1× working solution in H$_2$O before use.

**Tris-Acetate Electrophoresis (TAE) buffer (50× and 1×)**

One litre of 50× TAE stock solution was prepared by dissolving the following reagents in 750 ml of H$_2$O:

- Tris-base: 242 g
- Glacial acetic acid: 57.1 ml
- EDTA (0.5 M, pH 8.0): 100 ml

Total volume was made up to 1 litre with H$_2$O. The 50× TAE solution was sterilized by autoclaving and stored at 4 °C. The 1× TAE buffer working solution was prepared by diluting 200 ml of 50× TAE buffer (stock solution) in 10 litres of H$_2$O. The 1× TAE buffer was stored at room temperature.
2.1.4.4 Sample preparation buffers

Orange G loading buffer (10×)
Orange G gel loading buffer (50 ml) was prepared by dissolving the following reagents in H₂O:
- Sucrose: 20 g
- Bromophenol blue: 0.1 g

The gel buffer dispensed into 1 ml aliquots and stored at -20 °C.

Pre-stain gel loading buffer (6×) containing SYBR™ Green
Gel loading buffer (20 ml) was prepared by dissolving the following reagents in H₂O:
- Sucrose: 7 g
- Bromophenol blue: 0.05 g

The gel loading buffer was filter sterilized and stored at 4 °C. A 15 μl aliquot of 1:100 diluted SYBR™ Green solution was added to 985 μl of gel loading buffer before use.

SDS-PAGE sample loading buffer (3×)
100 mM Tris (pH 6.8)
4% (w/v) SDS
0.2% (w/v) Bromophenol blue
20% (v/v) glycerol
200 mM DTT

SDS sample loading buffer was stored at -20 °C.

Tris lysis buffer
50 mM Tris (pH 7.0)
250 mM NaCl
1.0% (w/v) Triton X-100

Tris lysis buffer was stored at -20 °C. A 20 μl aliquot of 100 mM PMSF was added to 20 ml of ice-cold lysis buffer before use.
2.1.5 Equipment

Centrifuges
Centrifugation of tubes with volumes up to and including 2 ml was carried out at either 4 °C or room temperature in an Eppendorf Centrifuge 5417C (Eppendorf, Germany) with a maximum speed of 25,000 g (14,000 rpm). Centrifugation of 15 ml and 50 ml tubes was carried out at either 4 °C or 20 °C in an Eppendorf Centrifuge 5804R (Eppendorf, Germany) with a maximum speed of 4,500 g (5,000 rpm).

Electrophoresis

DNA electrophoresis
Agarose gel electrophoresis was carried out in a Bio-Rad Wide Mini-Sub® Cell GT electrophoresis tank (Bio-Rad Laboratories, USA) using a Bio-Rad Power-Pac 300 power pack (Bio-Rad Laboratories, USA). Agarose gels were visualised using the LAS-3000 Intelligent DarkBox (FujiFilms, Tokyo, Japan). Gel images are shown with inverted white background. Digital images were edited using Adobe® Photoshop Elements Version 6.0 (Adobe Corporation, USA).

SDS-PAGE
SDS-PAGE was carried out using a Bio-Rad Mini-PROTEAN Tetra Cell electrophoresis tank (Bio-Rad Laboratories, USA) powered by a Bio-Rad Power-Pac 300 (Bio-Rad Laboratories, USA). If proteins samples were not transferred to PVDF membranes, then the proteins in the gel were stained using Coomasie brilliant blue R450. Stained gels were visualised using the LAS-3000 Intelligent DarkBox (FujiFilms, Tokyo, Japan) Digital images were edited using Adobe® Photoshop Elements Version 6.0 (Adobe Corporation, USA).

Western blot transfer
Transfer of protein samples separated by SDS-PAGE to PVDF membranes was carried out using a Bio-Rad Mini Trans-Blot cell powered by a Bio-Rad Power-Pac 300 (Bio-Rad Laboratories, USA).

Flow cytometer
Analysis of fluorescently labelled lymphocytes and bacteria was done using a BD FACS Canto II flow cytometer (BD Biosciences, USA).
**Fluorescence Microscope**
Bacteria expressing GFP and mouse tissue sections were imaged using a Nikon Ti-E inverted microscope with Nikon A1 Si spectral detector confocal system running NIS-C Elements software.

**Illuminator Box**
The excision of DNA from agarose gel was carried out using a Safe Imager® Transilluminator (Invitrogen, USA).

**pH Meter**
The pH of buffers and solutions was measured using an Ultrabasic Benchtop pH Meter (Denver Instrument Company, USA).

**Plate reader**
Measurements of optical density and fluorescence in 96 well format was conducted using a POLARstar Omega plate reader (BMG Labtech, Germany).

**Thermocycler**
PCR was completed in a Mastercycler Gradient S thermocycler (Eppendorf, Germany).

**Scanning Electron Microscope**
Bacteria stained with osmium and sputter-coated with palladium were visualized using a Zeiss 1555 VP-FESEM scanning electron microscope. (Zeiss, USA).

**Sonication**
Bacterial cell sonication was completed using a Sonicator® Ultrasonic Processor XL (Misonix Corporation, New York, USA).

**Spectrophotometers**
Measurements of optical density were conducted using a Shimadzu UV-1601 UV-VIS Spectrophotometer (Shimadzu Corporation, Japan). Measurements of DNA concentrations were conducted using a Micro-Volume UV-Vis Spectrophotometer or the NanoDrop 2000 (Thermo Scientific, USA).
**TissueLyser**

Stomach tissue collected from mice was homogenized by a TissueLyser with a TissueLyser adapter set (24-tubes) (Qiagen, Germany). One TissueLyser single bead dispenser (5 mm) was used in a 2 ml microcentrifuge tube.
2.2 General methods

2.2.1 Bacterial culture

2.2.1.1 E. coli growing conditions

_E. coli_ strains were cultured either on LB agar or in LB broth and incubated under aerobic conditions overnight at 37 °C. Antibiotics to select for recombinants were added to the media when required.

2.2.1.2 H. pylori growing conditions

The _H. pylori_ strains used in this study were routinely cultured at 37 °C under microaerobic conditions on Columbia blood agar (CBA) plates containing 5% horse blood and Dent’s antibiotic supplement (Oxoid). _H. pylori_ liquid culture: Bacteria were grown in BHI medium, Brucella Broth or HI medium supplemented with 10% Newborn Calf Serum (NCS) and Dent’s antibiotic supplement in upright 75 cm vented tissue culture flasks. Cultures were inoculated with bacteria suspended in PBS to give a starting OD$_{600}$ of 0.05, and grown under microaerobic conditions at 37 °C and 120 rpm. Microaerobic conditions were generated by incubating plates in BD GasPak™ EZ containers with Campy gas generating sachets (BD, USA) or by incubating plates in sealed jars using the Anoxomat™ MarkII system (Mart Microbiology B.V., The Netherlands) after one atmosphere replacement using a gas composition of N$_2$:H$_2$:CO$_2$ equal to 85:5:10. All liquid culture assays were conducted using the Anoxomat system. Antibiotics and media supplements were added to the media as required.

2.2.1.3 Culture storage

For short-term storage, _E. coli_ cultures were stored at 4 °C on LB agar. _H. pylori_ cultures are not suitable for short-term storage and therefore all _H. pylori_ strains were stored in glycerol stock at -80 °C once the recombination event was confirmed with minimal passage. For long term storage of _E. coli_ and _H. pylori_ cultures, glycerol stocks were made and stored at -80 °C. Glycerol stocks of _E. coli_ consisted of 1 ml of overnight _E. coli_ broth culture and 500 μl of sterile glycerol (final concentration of 33% v/v). Glycerol stocks of _H. pylori_ was prepared by the addition of a heavy loopful of bacteria harvested from 1-2 day old growth on CBA plates and resuspended in BHI media supplemented with glycerol (final concentration of 20% v/v).
2.2.2 General DNA manipulation procedures

Standard cloning and DNA analysis procedures were performed according to Sambrook and Russell (Sambrook and Russell, 2001).

2.2.2.1 Agarose gel electrophoresis

For routine DNA separation, 0.8% agarose gels were prepared with 1× TAE buffer. Gels were set in appropriate moulds for 30 min before transfer into Mini-Sub Cell gel tank (BioRad Laboratories) containing 1× TAE buffer. For pre-staining procedure, 1.5 μl of pre-stain gel loading buffer was mixed with 5 μl of DNA sample or 1 kb DNA ladder prior to loading into the wells. For post-staining procedure, 1.5 μl of orange gel loading buffer was used instead and after electrophoresis, gels were soaked for 20 min in 1× TAE buffer containing 1× SYBR ™ Safe stain. Unless otherwise stated, all gels were electrophoresed in 1× TAE buffer at 90 V for 50 min and visualised using the LAS-3000 (FujiFilms) and accompanying Image reader v2.2 software.

2.2.2.2 DNA purification

Purification of PCR products from solutions and agarose gels

Polymerase chain reaction (PCR) products were purified using PureLink™ PCR Purification Kit according to the manufacturer’s instructions. In brief, this involved the addition of a binding buffer containing isopropanol to the sample to facilitate binding of double stranded DNA to a silica-based membrane, followed by a washing step to remove impurities and final elution of DNA in low salt elution buffer. The PureLink™ Quick Gel Extraction Kit was used to purify DNA fragments from agarose gels according to the manufacturer’s instructions. After an initial gel solubilisation step, the purification involved was similar to the procedure for the PureLink™ PCR Purification Kit. All purified DNA fragments were run on a 0.8% agarose gel to ensure integrity of DNA following the purification procedure. Purified DNA was stored at -20 °C until use.

Plasmid DNA preparation

Plasmid DNA was extracted from broth based cultures of *E. coli* by using the PureLink™ Quick Plasmid Miniprep Kit as per the manufacturer’s instructions. Briefly, overnight bacterial culture (5 ml) was centrifuged at 4,000 g for 10 min. The cell pellet was resuspended in Buffer 1 containing RNase A to degrade RNA. The cells were lysed using alkaline/SDS method and clarified lysate (supernatant) was collected by
centrifugation at maximum speed for 10 min. The purification of plasmid DNA was based on a silica membrane column that selectively binds plasmid DNA and eluted in TE buffer.

**Genomic DNA preparation**
Genomic DNA was extracted from *H. pylori* grown on CBA plates for 1-2 days. Bacteria were harvested using a sterile loop and resuspended in 500 μl sterile saline. Genomic DNA was extracted using the PureLink™ Genomic DNA Kit as described in the manufacturer’s protocol. The purification procedure involved a similar methodology as used in the plasmid DNA preparation, with the alkaline/SDS cell lysis method replaced by Proteinase K digestion in the presence of guanidine hydrochloride, which inactivates nuclease and enhances binding of DNA to the column.

**Measurement of DNA concentration using spectrophotometry**
DNA concentration was determined by spectrophotometry using the NanoDrop 2000 according to the manufacturer’s protocol.

**2.2.2.3 PCR**

**Standard PCR**
Standard polymerase chain reactions (PCRs) were performed using different DNA polymerases, *Taq*, *MyTaq*, or *Pwo* Superyield DNA polymerase, with buffers supplied by the enzyme manufacturer. The components and reaction conditions of a standard PCR are listed in Table 2-6 and Table 2-7, respectively.

All dehydrated primers were reconstituted in ultra-pure H$_2$O to a stock concentration of 100 μM and working concentration of 2.5 μM. All primers were stored at -20 °C, and kept on ice when in use. All PCR reactions were performed using a Mastercycler Gradient S thermocycler (Eppendorf). PCR products were analysed by agarose gel electrophoresis. The amplification of different DNA molecules often required optimization of thermocycling conditions or PCR components. To the increase the specificity of primer-template hybridisation, thermal cycling conditions were optimized using gradient PCR to simultaneously test different annealing temperatures for maximum DNA amplification. Components of PCR reactions varied during optimization steps included: quantity of DNA template and addition of DMSO to overcome secondary structures.
### Table 2-6: Components of standard PCR using Taq, MyTaq or Pwo Superyield DNA polymerase

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Final concentration/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Buffer* (5-10×)</td>
<td>10 - 5</td>
<td>1×</td>
</tr>
<tr>
<td>dNTPs mix (2 mM)</td>
<td>5</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>DMSO+</td>
<td>2.5</td>
<td>5%</td>
</tr>
<tr>
<td>Forward primer (2.5 μM)</td>
<td>5</td>
<td>0.25 μM</td>
</tr>
<tr>
<td>Reverse primer (2.5 μM)</td>
<td>5</td>
<td>0.25 μM</td>
</tr>
<tr>
<td>DNA polymerase (5 U/μl)</td>
<td>0.5</td>
<td>2.5 U</td>
</tr>
<tr>
<td>DNA template</td>
<td>-</td>
<td>Variable</td>
</tr>
<tr>
<td>Made up with Ultra-pure H₂O to</td>
<td>50 μl</td>
<td></td>
</tr>
</tbody>
</table>

*MyTaq has a 5× reaction buffer.
+DMSO was only added to Pwo Superyield DNA polymerase PCR reactions when necessary.

### Table 2-7: Thermal cycling conditions for standard PCR

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time (s)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>45-60 °C*</td>
<td>30</td>
<td>28-35</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>60+</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>Hold^</td>
<td>10 °C</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*The annealing temperature varied according to the melting temperature of primers used for PCR.
+The extension time varied according to the length of the PCR product being amplified. Generally, an extension period of 60 s was provided per kb of PCR product to be amplified.
^PCR product was held at this temperature for up to 12 h.

### Colony PCR

The colony PCR was performed to identify *E. coli* recombinants. Bacteria were sampled from a colony of cells on an LB agar plate using a bevelled, sterile toothpick and were first inoculated onto a second agar plate for future reference and then transferred directly into the PCR mix on ice. To screen for recombinant *H. pylori*, genomic DNA was extracted and used as template in the PCR mix. The components of the colony PCR were the same as those for a standard PCR except that Taq polymerase was routinely used, the reaction was reduced to 20 μl and the initial denaturing step was extended to
600 s. The components and reaction conditions of a colony PCR are listed in Table 2-8 and Table 2-9, respectively.

**Table 2-8 Components of colony PCR using *Taq* or *MyTaq* DNA polymerase**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Final concentration/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Buffer* (5-10×)</td>
<td>4 - 2</td>
<td>1×</td>
</tr>
<tr>
<td>dNTPs mix (2 mM)</td>
<td>2</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Forward primer (2.5 μM)</td>
<td>2</td>
<td>0.25 μM</td>
</tr>
<tr>
<td>Reverse primer (2.5 μM)</td>
<td>2</td>
<td>0.25 μM</td>
</tr>
<tr>
<td><em>Taq</em> DNA polymerase (5 U/μl)</td>
<td>0.2</td>
<td>1 U</td>
</tr>
<tr>
<td>DNA template</td>
<td>-</td>
<td>Variable</td>
</tr>
<tr>
<td>Made up with Ultra-pure H₂O to</td>
<td>20 μl</td>
<td></td>
</tr>
</tbody>
</table>

* *MyTaq* has a 5× reaction buffer.

**Table 2-9 Thermal cycling conditions for colony PCR**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time (s)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>96 °C</td>
<td>600</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>45-60 °C*</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>60+</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>Hold^</td>
<td>10 °C</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*The annealing temperature varied according to the melting temperature of primers used for PCR.

+The extension time varied according to the length of the PCR product being amplified. Generally, an extension period of 60 s was provided per kb of PCR product to be amplified.

^PCR product was held at this temperature for up to 12 h.

**Extension PCR (Exte PCR)**

The Extension PCR (Exte PCR) is a special version of the colony PCR used for screening for *H. pylori* recombinants when standard colony PCR reactions failed. The Exte PCR reaction components were the same as that of the colony PCR but the PCR program was modified to start with a low annealing temperature and a 300 s extension time for the first 10 cycles, which was then followed by 25 cycles where the annealing temperature was 55 °C and the extension time increased by 20 s every cycle. This Exte PCR program is described in Table 2-10.
Table 2-10  Thermal cycling conditions for Extension PCR

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time (s)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>45 °C</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Extension</td>
<td>68 °C</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>55 °C</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Extension</td>
<td>68 °C</td>
<td>600^</td>
<td></td>
</tr>
<tr>
<td>Hold^</td>
<td>10 °C</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

^The extension increased by 20 s after each cycle to reach a final extension time of 1080 s.
^PCR product was held at this temperature for up to 12 h.

Short fusion PCR and Splicing by overlapping extension PCR (SOE PCR)

A variation of two techniques known as short fusion PCR (Shevchuk et al., 2004) and splicing by overlapping extension PCR (SOE PCR) (Heckman and Pease, 2007) was used to join two or more DNA fragments together in a two-step thermal cycling reaction. The technique used follows very closely to protocol described (Heckman and Pease, 2007) with the exception that in the second PCR reaction (SOE PCR), where the two DNA fragments are joined together, the PCR reactions lack primers for the first 10 cycles of the reaction and the annealing temperature is low. After the first 10 cycles, primers are added to the reaction and the annealing temperature is increased to a more stringent temperature as described (Shevchuk et al., 2004) (Table 2-11). High proof reading Pwo polymerase was employed in the first step PCR reaction, a standard PCR, to amplify each DNA fragment. AccuPrime™ Pfx was used in the second step PCR reaction, SOE PCR, to join the DNA fragments together. As a general rule, the internal primers were designed to have complementary sequences at their 5'-ends, resulting in PCR products I and II having a 20- to 30- complementary nucleotide overhang that can act as self-primers in the first 10 cycles of the SOE PCR reaction. In the first PCR step, two separate standard PCRs were performed to amplify two DNA fragments. The two PCR products were gel-purified and mixed at a ratio of 1:1, and used as templates in the second step PCR reaction. The SOE PCR reaction mixture is described in Table 2-12. These SOE PCR products were analysed using agarose gel electrophoresis.
Table 2-11  Thermal cycling conditions for second step, SOE PCR

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time (s)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>45 °C</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Extension</td>
<td>68 °C</td>
<td>60+</td>
<td></td>
</tr>
<tr>
<td>Hold*</td>
<td>68 °C</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>55 °C</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td>Extension</td>
<td>68 °C</td>
<td>60+</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>68 °C</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>Hold^</td>
<td>10 °C</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*The extension time varied according to the length of the PCR product being amplified. Generally, an extension period of 60 s was provided per kb of PCR product to be amplified.
*PCR product was held at this temperature until primers were added to the reaction.
^PCR product was held at this temperature for up to 12 h.

Table 2-12  Components of SOE PCR using AccuPrime™ Pfx DNA polymerase

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Final concentration/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>AccuPrime™ Pfx SuperMix</td>
<td>22.5</td>
<td>1×</td>
</tr>
<tr>
<td>PCR product I (~10 ng/μl)</td>
<td>1</td>
<td>10 ng</td>
</tr>
<tr>
<td>PCR product II (~10 ng/μl)</td>
<td>1</td>
<td>10 ng</td>
</tr>
<tr>
<td>Forward primer* (25 μM)</td>
<td>0.25</td>
<td>0.25 μM</td>
</tr>
<tr>
<td>Reverse primer* (25 μM)</td>
<td>0.25</td>
<td>0.25 μM</td>
</tr>
</tbody>
</table>

* Primers were added to the reaction after 10 cycles of the SOE PCR program.

2.2.2.4 Recombinant DNA cloning procedures

Restriction endonuclease digestions

Digestion of DNA with restriction endonucleases was performed to prepare DNA for cloning and to identify recombinant plasmids. The amount of DNA used for digestions was determined by comparing the staining intensity of the band corresponding to the DNA of interest to the staining intensity of the bands in the λ HindIII ladder, resolved on 0.8% agarose gel. The composition of the digestion reaction mixture used to prepare DNA for cloning is described in Table 2-13. The reaction mixture was incubated for 2-4 h at 37 °C. Digested DNA was run on 0.8% agarose gel and the DNA fragment of
interest was excised from the gel and purified using PureLink™ Quick Gel Extraction Kit or when appropriate DNA was purified from the digestion reaction directly using the PureLink™ PCR Purification Kit as described in 2.2.2.2. To identify recombinant plasmids a diagnostic restriction endonuclease digestion reaction mixture, described in Table 2-14, was prepared. The volume of digestion reaction was reduced to 20 μl and the reaction was incubated at 37 °C for only 1 h.

Table 2-13 Composition of restriction endonuclease digestion reactions

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Final concentration/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid DNA (~ 50 ng/μl)</td>
<td>10</td>
<td>~ 500 ng</td>
</tr>
<tr>
<td>PCR product (~ 5 ng/μl)</td>
<td>40</td>
<td>~ 200 ng</td>
</tr>
<tr>
<td>NEB Buffer* (10×)</td>
<td>5</td>
<td>1×</td>
</tr>
<tr>
<td>Restriction enzyme(s)*</td>
<td>0.5</td>
<td>1×</td>
</tr>
<tr>
<td>BSA (100×)^</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Made up with Ultra-pure H₂O to</td>
<td>50 μl</td>
<td></td>
</tr>
</tbody>
</table>

*NEB buffer and enzyme(s) was used according to manufacturer’s protocol.
^BSA was added into the reaction if required.

Table 2-14 Composition of diagnostic restriction endonuclease digestion reactions

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Final concentration/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA (~ 10 ng/μl)</td>
<td>2</td>
<td>~ 20 ng</td>
</tr>
<tr>
<td>NEB Buffer* (10×)</td>
<td>2</td>
<td>1×</td>
</tr>
<tr>
<td>Restriction enzyme(s)*</td>
<td>0.2</td>
<td>1×</td>
</tr>
<tr>
<td>BSA (100×)^</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Made up with Ultra-pure H₂O to</td>
<td>20 μl</td>
<td></td>
</tr>
</tbody>
</table>

*NEB buffer and enzyme(s) was used according to manufacturer’s protocol.
^BSA was added into the reaction if required.

**DNA blunting reactions**

DNA blunting reactions were performed when specific restriction endonuclease sites had to be removed from a plasmid to permit subsequent DNA manipulations or when a plasmid did not have suitable restriction endonuclease sites to facilitate cloning of the DNA fragment of interest. Purified PCR products and restriction endonucleases treated DNA fragments were incubated with T4 DNA polymerase and dNTPs to fill in
5′ overhangs and remove 3′ overhangs to generate blunt ends. The composition of the blunting reaction mixture is described in Table 2-15. The reaction mix was incubated at 12 °C for 12 min. Treated DNA was immediately purified using PureLink™ PCR Purification Kit as described in 2.2.2.2.

**Table 2-15 Composition of T4 DNA polymerase blunting reactions**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Final concentration/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid DNA (~ 50 ng/μl)</td>
<td>10</td>
<td>~ 500 ng</td>
</tr>
<tr>
<td>PCR product (~ 10 ng/μl)</td>
<td>40</td>
<td>~ 400 ng</td>
</tr>
<tr>
<td>NEB Buffer 2 (10×)</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>dNTPs mix (2 mM)</td>
<td>5</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>BSA (100×)</td>
<td>0.5</td>
<td>1×</td>
</tr>
<tr>
<td>T4 DNA polymerase</td>
<td></td>
<td>5 U</td>
</tr>
<tr>
<td>Made up with Ultra-pure H2O to</td>
<td>50 μl</td>
<td></td>
</tr>
</tbody>
</table>

**DNA dephosphorylation**

To prevent recircularization of cloning vectors, Calf Intestinal Alkaline Phosphatase (CIP) was used to remove the 5′ phosphates of restriction endonuclease treated plasmids. The composition of DNA dephosphorylation reactions in described in Table 2-16. The reactions were incubated at 37 °C for 30 min. DNA was purified using PureLink™ PCR Purification Kit as described in 2.2.2.2.

**Table 2-16 Composition of DNA dephosphorylation reactions**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Final concentration/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested plasmid DNA</td>
<td>40</td>
<td>~ 100 ng</td>
</tr>
<tr>
<td>NEB Buffer* (10×)</td>
<td>5</td>
<td>1×</td>
</tr>
<tr>
<td>CIP</td>
<td></td>
<td>10 U</td>
</tr>
<tr>
<td>Made up with Ultra-pure H2O to</td>
<td>50 μl</td>
<td></td>
</tr>
</tbody>
</table>

*CIP is compatible with all NEB buffers, therefore if the restriction endonucleases used in the previous cloning step could be heat inactivated, following heat inactivation, 1 μl of CIP was directly added to the reaction.
Chapter 2: General materials and methods

DNA ligations
To join DNA insert fragments and linearized vector DNA, a ligation reaction using T4 DNA ligase was performed. An insert to vector DNA ratio of 5:1 was used in ligation reactions. Ligation reactions of DNA fragments with either compatible overhanging termini, ‘sticky-ends’, or with blunt ends were incubated for 2 h at 18 °C, and were subsequently used to transform chemically competent *E. coli* strain DH5α cells. The composition of DNA ligation reactions is described in Table 2-17.

Table 2-17 Composition of DNA ligation reactions

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Final concentration/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear plasmid DNA (~ 30 ng/μl)</td>
<td>2</td>
<td>~ 60 ng</td>
</tr>
<tr>
<td>Insert (~ 10 ng/μl)</td>
<td>8</td>
<td>~ 80 ng</td>
</tr>
<tr>
<td>T4 DNA Ligase Buffer (10×)</td>
<td>2</td>
<td>1×</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1</td>
<td>40 U</td>
</tr>
<tr>
<td>Made up with Ultra-pure H₂O to</td>
<td>20 μl</td>
<td></td>
</tr>
</tbody>
</table>

Occasionally PCR products were cloned directly into a linearized plasmid containing a single 3′-terminal thymidine at both ends, such as the pGEM®-T Easy vector. The purified PCR DNA fragment, generated by the high proofreading *Pwo* DNA polymerase, was modified using an A-Tailing procedure to add a single 3′ terminal adenine on both ends of the DNA fragment. The components of the A-Tailing reaction are listed in Table 2-18. The reaction was incubated at 70 °C for 30 min. A 2 μl aliquot of this reaction mixture was used as the insert and combined with 1 μl (50 ng) of pGEM®-T Easy vector in a standard ligation reaction described above.

Table 2-18 Composition of A-Tailing reaction

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Final concentration/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product (~ 10 ng/μl)</td>
<td>7</td>
<td>~ 70 ng</td>
</tr>
<tr>
<td>PCR Buffer (10×)</td>
<td>1</td>
<td>1×</td>
</tr>
<tr>
<td>dNTPs mix (2 mM)</td>
<td>1</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/μl)</td>
<td>1</td>
<td>5 U</td>
</tr>
<tr>
<td></td>
<td>10 μl</td>
<td></td>
</tr>
</tbody>
</table>
2.2.2.5 E. coli transformation

Competent E. coli DH5α cells were prepared using the standard chemical method with RbCl (Sambrook and Russell, 2001). The ligation reaction (20 μl) was gently mixed with 100 μl of competent E. coli cells and chilled on ice for 30 min. Cells were heat shocked at 42 °C for 30 s, then returned to chill on ice for 2 min. A 900 μl aliquot of warm SOC broth was added to the cell suspension and incubated aerobically for 1 h at 37 °C with constant shaking at 220 rpm. The transformation culture (200 μl and the remainder) was spread onto LB agar plates supplemented with appropriate antibiotics and incubated overnight at 37 °C.

2.2.2.6 Characterization of recombinant E. coli

Bacteria harbouring plasmids were selected for on LB agar supplemented with the appropriate antibiotics. The recombinant plasmids constructed using pHSG576 or pGEM®-T Easy were selected by blue/white screening (Sambrook and Russell, 2001). The white colonies were subcultured and characterized by colony PCR and enzyme mapping to confirm the presence of the insert. Recombinant plasmids based on other cloning vectors were identified using colony PCR and restriction enzyme mapping as described in sections 2.2.2.3 and 2.2.2.4 respectively.

2.2.3 DNA manipulation in H. pylori

2.2.3.1 H. pylori transformation

Natural transformation of H. pylori was performed on an agar surface according to the methods described (Wang et al., 1993). In brief, H. pylori recipient strains were inoculated from a -80 °C glycerol stock onto CBA agar plates and incubated for 2-3 days. Bacteria were passaged once onto fresh CBA plates and incubated for 24 h. A loopful of bacteria was plated onto the surface of a new CBA plate, containing DENT supplement, to form a concentrated area of bacteria, 8-10 mm in diameter, and incubated for a further 5 h. Following this incubation, a 5-10 μl aliquot of DNA, (0.5-2 μg), resuspended in TE buffer was mixed into the bacterial spot, maintaining a diameter of 10-15 mm, and the plate was incubated for another 20-24 h under microaerobic conditions. Bacterial cells incubated with DNA were streaked onto fresh CBA plates supplemented with the appropriate antibiotics. Growth on selective medium was observed approximately 3-5 days post-transformation.
2.2.3.2 Characterization of recombinant *H. pylori*

*H. pylori* recombinants were first selected on CBA plates supplemented with the appropriate antibiotics. Some antibiotic resistant colonies underwent a second antibiotic selection step (streptomycin and chloramphenicol) if the recombination was based on the counterselectable streptomycin susceptibility determinant (Dailidiene et al., 2006). Positive recombinants were further confirmed by screening with PCR based analyses and DNA sequencing, as described in sections 2.2.2.3 and 2.2.5 respectively.

2.2.3.3 Recombination based on the streptomycin counterselection

The main *H. pylori* strains used in this study, X47 and 26695, do not readily take up and replicate plasmids, making plasmids an unfeasible way of introducing new or additional copies of genes or DNA into these strains. A strategy used in this study to modify *H. pylori* chromosomal DNA to introduce new genes or create markerless mutations involves the use of counterselectable streptomycin susceptibility determinant (Dailidiene et al., 2006). In this study, the selection cassette, *rpsL-cat* (Styer et al., 2010) is used for this purpose. This system utilizes the wild-type of the *rpsL* gene from *C. jejuni*, which encodes for streptomycin sensitivity and is dominant over the mutant form which shows streptomycin resistance (Dailidiene et al., 2006), and the *cat* gene that provides resistance to chloramphenicol. The *rpsL* gene in the *rpsL-cat* cassette is 18% divergent from the *rpsL* in *H. pylori* and can reduce the occurrence of gene conversion between two *rpsL* genes in the chromosome.

To modify a region in the *H. pylori* genome, specific example: insert a copy of *gfpmut2* into the HP1277 gene, (ref 3.2.4) A streptomycin resistant *H. pylori* strain is transformed with DNA containing *rpsL-cat* flanked on either side by at least 500 bp of DNA sequence from the target locus, and *H. pylori* recombinants are selected for on CBA plates containing 10 μg/ml chloramphenicol. Recombinant strains are screened by colony PCR for insertion of *rpsL-cat* into HP1277. Correct recombinant *H. pylori* strains are termed recipient strains and are Cm$^r$ and Str$^\s$. The recipient strain is then transformed with DNA containing the *gfpmut2* similarly flanked on either side by at least 500 bp of DNA sequences from the target locus, and recombinant *H. pylori* clones are selected for on CBA plates containing 10 μg/ml streptomycin. As gene conversion between two *rpsL* genes in the chromosome can occur, colonies isolated on streptomycin plates are patched onto new streptomycin plates and replica plated onto plates containing chloramphenicol. Colonies that fail to grow on chloramphenicol plates
are chosen from the streptomycin plates and analysed by colony PCR for appropriate replacement of the \textit{rpsL-cat} cassette with \textit{gfp}mut2.

### 2.2.4 General proteins analysis procedures

#### 2.2.4.1 Sample preparation

Bacterial whole cell lysates were prepared by resuspending bacteria grown on CBA plates for 24 h in PBS buffer. Cells were pelleted, washed a second time with PBS, resuspended in ice-cold Tris lysis buffer and incubated on ice for 15 min. Bacterial cells grown in liquid culture were first pelleted, and the media was decanted. Cells were resuspended and washed in PBS twice before a final resuspension in ice-cold Tris lysis buffer. Following the 15 min incubation on ice, samples were sonicated for 10 s. Insoluble cell debris was removed by centrifugation at 13,000 rpm form 10 min at 4 °C and clarified supernatant was dispensed in 100 μl aliquots and stored at -20 °C. The protein concentration of each sample was measured using Bradford reagent and protein concentrations were standardized to 1 μg/μl. To prepare the sample for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), cell lysates were mixed with 3× SDS-PAGE sample loading buffer and incubated at 95 °C for 10 min.

#### 2.2.4.2 SDS-PAGE and electroblotting

Protein samples, 10-20 μg per lane, were loaded onto 10% Tris-HCl polyacrylamide gels using a mini-gel apparatus (Bio-Rad Laboratories) according to the manufacturer’s instructions. Gels were electrophoresed at room temperature in 1× SDS-PAGE running buffer at 90 V for the first 20 min, and subsequently at 180 V until the dye front had reached the bottom of the resolving gel. After electrophoresis, the samples were electroblotted to polyvinylidene fluoride (PVDF) membrane (0.2 μm) by wet-transfer at 90 V, at 4 °C for 2 h. The BioRad electrotransfer apparatus was assembled and used according to manufacturer’s instructions (BioRad Laboratories). Transfer was verified by visual inspection of the transfer of pre-stained markers. The membrane was washed in deionized water three times for 5 min each and allowed to dry completely, and stored at room temperature until the immunodetection procedure.
2.2.4.3 Immunoblotting

Protein samples separated on SDS-PAGE were transferred onto 0.2 μm PVDF membrane using a Bio-Rad transblot system. The dry membrane was rehydrated with methanol and washed once in PBST for 5 min. The membrane was blocked with blocking buffer, PBST containing 2% bovine serum albumin (BSA) (w/v), for 1 h at room temperature. The blocking solution was decanted, and a solution of blocking buffer containing the primary antibody was added and incubated with the membrane for 1 h. The membrane was rinsed for 2 × 5 min and 3 × 15 min with PBST and then incubated with blocking buffer containing the secondary antibody conjugated to horseradish peroxidise (HRP) at a dilution of 1:10000 for 1 h at room temperature. The membrane was rinsed again, for 2 × 5 min and 3 × 15 min with PBST, and detection of the secondary HRP conjugate was accomplished with chemiluminescence detection by using Chemiluminescent Peroxidase Substrate-3 (Sigma) and LAS 3000 Intelligent dark box (Fujifilm)(software Image reader LAS 3000 V2.2).

2.2.5 DNA Sequencing

2.2.5.1 DNA sequencing reactions

The recombinant plasmids and the genomic DNA of recombinant H. pylori strains were further characterized by sequencing. BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystem, USA) was used according to the manufacturer’s instructions for all sequencing reactions. This sequencing kit is based on the dye terminator sequencing method which uses fluorescently labelled dideoxy chain terminators. For the sequencing of the recombinant plasmids, plasmid DNA was used directly as the template for the DNA sequencing reaction. For the sequencing of specific regions of genomic DNA isolated from recombinant H. pylori, a standard PCR was used to amplify the region of interest, and the cleaned PCR product was used as the DNA template for DNA sequencing reactions. The amount of template DNA added to the reaction mix varied depending on the length, purity and nature of the DNA template use. The composition of the DNA sequencing reaction is shown in Table 2-19. Thermal cycling conditions for DNA sequencing reaction are shown in Table 2-20. DNA sequencing reactions were cleaned and purified using ethanol/EDTA precipitation and were processed at the Lotterywest Biomedical Facility (LBF, Royal Perth Hospital) using ABI prism 3730 (48 capillary) or ABI prism 3730XL (96 capillary) DNA sequencers.
### Table 2-19  Composition of 20 μl DNA sequencing reaction

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
<th>Final concentration/reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>x</td>
<td>30 ng</td>
</tr>
<tr>
<td>BigDye® Terminator ready reaction mix v3.1 (2.5×)</td>
<td>4</td>
<td>1×</td>
</tr>
<tr>
<td>BigDye Sequencing Buffer (5×)</td>
<td>2</td>
<td>1×</td>
</tr>
<tr>
<td>Primer (3.2 μM)</td>
<td>1</td>
<td>0.16 μM</td>
</tr>
<tr>
<td>Made up with Ultra-pure H₂O to</td>
<td>20 μl</td>
<td></td>
</tr>
</tbody>
</table>

*DNA concentration of template DNA was estimated using spectrophotometry at 260 nm (see section 2.2.2.2).

### Table 2-20  Thermal cycling conditions for sequencing reactions

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature*</th>
<th>Time (s)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>600</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>50-55 °C*</td>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td>Extension</td>
<td>60 °C</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>Hold^</td>
<td>4 °C</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*The rapid thermal ramp was 1 °C /s when temperature changed.

*The annealing temperature varied according to the melting temperature of primers used for PCR.

^PCR product was held at this temperature for up to 12 h.

#### 2.2.5.2  Ethanol/EDTA precipitation

DNA sequencing reactions were cleaned and purified as follows. DNA was precipitated by the addition of 5 μl of 125 mM EDTA (pH 8.0) and 60 μl of 100% analytical grade ethanol to the 20 μl sequencing reactions. Samples were mixed by inversion 4 times and allowed to stand at room temperature for 15 min before centrifugation at 2,500 g for 30 min. The supernatant was carefully removed and the pellet was rinsed with 70 μl of 70% ethanol. The sample was centrifuged again at 2,500 g for 30 min. The supernatant was carefully removed and the pellet was allowed to air-dry. The dry DNA pellet was sent to the Sequencing Unit of LBF.
2.3 Animal experimental procedures

2.3.1 Animals

*Helicobacter* free, female C57BL/6J mice, provided by the Animal Resource Centre, Australia, were used in this study. Mice, 6-8 weeks of age at the start of each experiment, were housed in groups of 3 to 6 in micro-isolator caging in the Animal Care Facility located in M-block, QE II Medical Centre, University of Western Australia. The mice were fed a pelleted rodent chow and were provided acidified drinking water unless otherwise stated for the duration of the experiments. Studies were performed with approval from the UWA Animal Ethics Committee (approval no. RA07/100/598 and RA03/100/723).

2.3.2 Bacteria

*H. pylori* strains used for infection studies were cultured at 37 °C on CBA plates and incubated under microaerobic conditions. The bacteria were harvested after 24 h of growth and suspended in Brucella broth. The morphology and motility of the *H. pylori* bacteria were examined by Gram stain and phase microscopy prior to inoculation. Mice were orally challenged once with 0.2 ml bacterial inoculum containing $1 \times 10^9$ colony-forming units (CFU) of *H. pylori* per ml.

2.3.3 Selective plates for isolation of *H. pylori* from murine stomach tissue

Selective plates were used for the isolation of *H. pylori* strains from murine stomach tissue. The plates were prepared as CBA plates with addition of the following antibiotics: DENT supplement, bacitracin, nalidixic acid and polymyxin B (Table 2-5).
2.3.4 Evaluation of the infection

Mice were sacrificed at the scheduled time intervals. Stomachs were removed and opened along the minor curvature. The non-mucosal, squamous forestomach was discarded and the stomach content gently removed with a sterile loop. The quantitative bacterial culture was carried out as described (Akada et al., 2003). In brief, stomach tissue was roughly cut into smaller pieces and homogenized in 500 μl of either BHI or HI broth using a tissue lyser (Qiagen). A 50 μl aliquot of the homogenized tissue was added to 450 μl of broth and diluted by serial dilution. Aliquots of 100 μl of the neat, $1 \times 10^2$ and $1 \times 10^3$ dilutions of the stomach homogenate were spread onto selective plates and cultured for 4 days. Colonies were identified as *H. pylori* by their characteristic morphology and by standard criteria such as Gram stain and biochemical tests (urease, catalase, and oxidase). The number of colonies was counted and CFU/stomach was calculated to determine the bacterial load. *H. pylori* colonies isolated from murine stomachs were characterized and stored in glycerol stock at -80 °C.

2.3.5 Tetracycline supplementation in drinking water

When required, mice received tetracycline in their drinking water containing 5% sucrose. Water containing 5% sucrose was sterilized by autoclaving prior to the addition of tetracycline. Tetracycline stock solutions (1000×) were filter sterilized and stored at -20 °C. Drinking water was prepared and changed every three days and water bottles containing tetracycline were protected from light. Mice commenced tetracycline supplementation 24 h before oral challenge with *H. pylori*. 
3 Plasmid and strain construction

3.1 Plasmids and strains used in this study

All the plasmids used in this study are listed in Table 3-1. The plasmids used for construction of the *H. pylori* tet-system were developed based on the cloning vector pBlu-SK-alt (see section 2.1.2.1). The plasmids used to make the second generation of CGT knockout constructs are based on the pGEM®-T Easy cloning vector (see section 2.1.2.1). Plasmids pWH1925 BD and pWH1925 r2 (Scholz et al., 2004) served as DNA templates for the amplification of *tetR* and *revtetR* respectively, and were kindly provided by Prof. Wolfgang Hillen (Nürnberg, Germany). Plasmid pONDG was the DNA template for amplification of *gfp*mut2 gene to construct *tet*-regulated GFP expression constructs and was kindly provided by Dr. Ji Kevin Li, a past PhD student, who received the *gfp*mut2 gene in the form of pIVET-Hpi from by Prof. Francis Megraud (Bordeaux, France) (Angelini et al., 2004). Plasmid pENT-RC, made by Ondek Pty. Ltd. (Carola Schwan), was used as a source for the *rpsL-cat* counterselection cassette (Styer et al., 2010). Plasmid pMdaB was made by Ondek Pty. Ltd. (Julia Satiaputra) and was used to generate plasmids containing the *ptetR* constructs. Plasmid pHSG576 is a low copy plasmid kindly provided by Dr. Charlene Kahler (Perth, Australia) (Takeshita et al., 1987), and pHRdx is a derivative of pHSG576, made by Ondek Pty. Ltd. (Dr. Tobias Schoep), containing regions of homology to the *rdxA* locus (HP0954).

All the strains used in this study are listed in Table 3-2. *E. coli* DH5α was used as the host strain for routine molecular cloning. *H. pylori* X47-2AL (X47) was used as the host strain for development of the *tet*-inducible system. The use of the counterselectable streptomycin susceptibility (*rpsL*-based) system requires a host strain that possesses a streptomycin-resistant phenotype. The *H. pylori* X47 host strain is a naturally streptomycin-resistant strain (Dailidiene et al., 2006) and no modifications to this strain were required. Two *H. pylori* recipient strains used in this study, X47 *rdxA::rpsL-cat* and X47 *mdaB::rpsL-cat* were made by Ondek Pty. Ltd. (Dr. Tobias Schoep and Julia Satiaputra respectively).

Primers used in this study are shown in Table 3-3.
<table>
<thead>
<tr>
<th>AD #</th>
<th>Plasmid name</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pHSG576</td>
<td>low copy plasmid</td>
<td>(Takeshita et al., 1987)</td>
</tr>
<tr>
<td></td>
<td>pHrdx</td>
<td>derivative of pHSG576, contains regions of homology to regions flanking HP0954 ORF, contains multiple cloning site</td>
<td>Ondek Pty. Ltd.</td>
</tr>
<tr>
<td></td>
<td>pHBlu-SK-alt</td>
<td>derivative of pBluescript SK (-) lacking XhoI and SalI restriction sites</td>
<td>Ondek Pty. Ltd.</td>
</tr>
<tr>
<td></td>
<td>pHBlu-BI</td>
<td>derivative of pHBlu-SK-alt, contain regions of homology to HP0072 and HP0071, contains multiple cloning site</td>
<td>(Benghezal et al., 2010)</td>
</tr>
<tr>
<td>pAD301</td>
<td>pTrpA</td>
<td>derivative of pHBlu-SK-alt, contain regions of homology to HP1277, contains multiple cloning site</td>
<td>This work</td>
</tr>
<tr>
<td>pAD302</td>
<td>pTrpA-RCAT</td>
<td>derivative of pTrpA, rpsL-cat</td>
<td>This work</td>
</tr>
<tr>
<td>pAD303</td>
<td>pGltDH</td>
<td>derivative of pHBlu-SK-alt, contain regions of homology to HP0379 and HP0380, contains multiple cloning site</td>
<td>This work</td>
</tr>
<tr>
<td>pAD304</td>
<td>pGltDH-RCAT</td>
<td>derivative of pGltDH, rpsL-cat</td>
<td>This work</td>
</tr>
<tr>
<td>pAD305</td>
<td>pHdapB</td>
<td>derivative of pHSG576, contains regions of homology to HP0509 and HP0511, contains multiple cloning site</td>
<td>This work</td>
</tr>
<tr>
<td>pAD306</td>
<td>pHdapB-RCAT</td>
<td>derivative of pHdapB, rpsL-cat</td>
<td>This work</td>
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<tr>
<td>pWH1925 BD</td>
<td>template for tetR</td>
<td>template for tetR</td>
<td>(Schnappinger et al., 1998)</td>
</tr>
<tr>
<td>pWH1925 r2</td>
<td>template for revtetR</td>
<td>template for revtetR</td>
<td>(Scholz et al., 2004)</td>
</tr>
<tr>
<td>pONDG</td>
<td>template for gfpmut2</td>
<td>template for gfpmut2</td>
<td>Made by Dr. Ji Kevin Li</td>
</tr>
<tr>
<td>pENT-RC</td>
<td>source of rpsL-cat counterselection cassette</td>
<td>source of rpsL-cat counterselection cassette</td>
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</tr>
<tr>
<td>pMdaB</td>
<td>derivative of pHBlu-SK-alt, contain regions of homology to HP0630 and HP0631, contains multiple cloning site</td>
<td>Ondek Pty. Ltd.</td>
<td></td>
</tr>
<tr>
<td>pMdaB-RCAT</td>
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<td>derivative of pMdaB, rpsL-cat</td>
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<tr>
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<tr>
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<td>derivative of pMdaB, PflaA-tetR</td>
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<tr>
<td>pAD311</td>
<td>pMdaB-p tetR5</td>
<td>derivative of pMdaB, PtaTaat-revtetR</td>
<td>This work</td>
</tr>
<tr>
<td>AD #</td>
<td>Plasmid name</td>
<td>Description</td>
<td>Source or reference</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------</td>
<td>--------------------------------------------------</td>
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<td>derivative of pTrpA, uPtetO1-GFP</td>
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<td>pAD401</td>
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<td>pAD404</td>
<td>pTrpA-uPtetO1-CGT</td>
<td>derivative of pTrpA, expresses CGT (HP0421) under uPtetO1</td>
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<tr>
<td>pAD405</td>
<td>pTrpA-uPtetO2-CGT</td>
<td>derivative of pTrpA, expresses CGT (HP0421) under uPtetO2</td>
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<td>pAD406</td>
<td>pTrpA-uPtetO3-CGT</td>
<td>derivative of pTrpA, expresses CGT (HP0421) under uPtetO3</td>
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<td>pAD407</td>
<td>pGltDH-uPtetO1-CGT</td>
<td>derivative of pGltDH, expresses CGT (HP0421) under uPtetO1</td>
<td>This work</td>
</tr>
<tr>
<td>pAD408</td>
<td>pGltDH-uPtetO2-CGT</td>
<td>derivative of pGltDH, expresses CGT (HP0421) under uPtetO2</td>
<td>This work</td>
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<tr>
<td>pAD409</td>
<td>pGltDH-uPtetO3-CGT</td>
<td>derivative of pGltDH, expresses CGT (HP0421) under uPtetO3</td>
<td>This work</td>
</tr>
<tr>
<td>pAD410</td>
<td>pHdapB-uPtetO1-CGT</td>
<td>derivative of pHdapB, expresses CGT (HP0421) under uPtetO1</td>
<td>This work</td>
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<tr>
<td>pAD411</td>
<td>pHdapB-uPtetO2-CGT</td>
<td>derivative of pHdapB, expresses CGT (HP0421) under uPtetO2</td>
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<tr>
<td>AD#</td>
<td>Plasmid name</td>
<td>Description</td>
<td>Source or reference</td>
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<tr>
<td>-------</td>
<td>-------------------------------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>pAD412</td>
<td>pTrpA-uPtetO1-CGT-HA</td>
<td>derivative of pTrpA, expresses CGT with C-terminal HA tag (HP0421) under uPtetO1</td>
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</tr>
<tr>
<td>pAD413</td>
<td>pTrpA-uPtetO2-CGT-HA</td>
<td>derivative of pTrpA, expresses CGT with C-terminal HA tag (HP0421) under uPtetO2</td>
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<td>pAD414</td>
<td>pTrpA-uPtetO3-CGT-HA</td>
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<td>pGltDH-uPtetO3-CGT-HA</td>
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<td></td>
<td>pGEM®-T Easy</td>
<td>TA cloning of vector</td>
<td>Promega, Australia</td>
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<td>pAD416</td>
<td>pCGT-Δ2</td>
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<td>This work</td>
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<td>pCGT-Δ2-RCAT</td>
<td>Derivative of pCGT-Δ2, contains counterselection cassette rpsL-cat</td>
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<td>pAD419</td>
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<td>Derivative of pCGT-Δ3, contains counterselection cassette rpsL-cat</td>
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<tr>
<td>AD#</td>
<td>Strain name</td>
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<td>Source or reference</td>
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<td></td>
<td><em>E. coli</em> strains</td>
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<tr>
<td></td>
<td>DH5α</td>
<td><em>fhuA2 ∆(argF-lacZ)U169 phoA glnV44</em> ∆(lacZ)M15 gvrA96 recA1 relA1 endA1 thi-1 hsdR17*</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>eAD1</td>
<td>DH5α pMdaB-ptetR5</td>
<td>PtaTaat drives expression of <em>revtetR</em></td>
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<tr>
<td>eAD2</td>
<td>DH5α pMdaB-ptetR6</td>
<td>PtaTaat drives expression of <em>tetR</em></td>
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<td><em>H. pylori</em> strains:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X47</td>
<td>wild-type strain, also known as X47-2AL, naturally resistant to streptomycin</td>
<td>(Ermak et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>X47 mdaB::rpsL-cat</td>
<td>rpsL-cat inserted between HP630 and HP631</td>
<td>Ondek Pty. Ltd.</td>
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<tr>
<td>hAD1</td>
<td>X47 mdaB::ptetR1</td>
<td>promoter-tetR1 inserted between HP630 and HP631</td>
<td>This work</td>
</tr>
<tr>
<td>hAD2</td>
<td>X47 mdaB::ptetR2</td>
<td>promoter-tetR2 inserted between HP630 and HP631</td>
<td>This work</td>
</tr>
<tr>
<td>hAD3</td>
<td>X47 mdaB::ptetR3</td>
<td>promoter-tetR3 inserted between HP630 and HP631</td>
<td>This work</td>
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<tr>
<td>hAD4</td>
<td>X47 mdaB::ptetR4</td>
<td>promoter-tetR4 inserted between HP630 and HP631</td>
<td>This work</td>
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<tr>
<td>hAD5</td>
<td>X47 mdaB::ptetR5</td>
<td>promoter-tetR5 inserted between HP630 and HP631</td>
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<td>hAD6</td>
<td>X47 mdaB::ptetR6</td>
<td>promoter-tetR6 inserted between HP630 and HP631</td>
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<tr>
<td>hAD7</td>
<td>X47 mdaB::ptetR7</td>
<td>promoter-tetR7 inserted between HP630 and HP631</td>
<td>This work</td>
</tr>
<tr>
<td>hAD8</td>
<td>X47 trpA::rpsL-cat</td>
<td>rpsL-cat inserted into HP1277</td>
<td>Made by Carola Schwan</td>
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<tr>
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<td></td>
<td>using pTrpA-RCAT</td>
</tr>
<tr>
<td>hAD9</td>
<td>X47 gltDH::rpsL-cat</td>
<td>rpsL-cat inserted between HP0379 and HP380</td>
<td>Made by Carola Schwan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>using pGltDH-RCAT</td>
</tr>
<tr>
<td>hAD10</td>
<td>X47 dapB::rpsL-cat</td>
<td>HP0510 is replaced with rpsL-cat</td>
<td>This work</td>
</tr>
<tr>
<td>hAD11</td>
<td>X47 trpA::uPtetO1-GFP</td>
<td>uPtetO1-GFP inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD12</td>
<td>X47 trpA::uPtetO2-GFP</td>
<td>uPtetO2-GFP inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD13</td>
<td>X47 gltDH::uPtetO1-GFP</td>
<td>uPtetO1-GFP inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD14</td>
<td>X47 gltDH::uPtetO2-GFP</td>
<td>uPtetO2-GFP inserted between HP0379 and HP380</td>
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<tr>
<td>hAD15</td>
<td>X47 dapB::uPtetO1-GFP</td>
<td>HP0510 is replaced with uPtetO1-GFP</td>
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<td>AD#</td>
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<td>hAD16</td>
<td>X47 dapB::uPtet02-GFP</td>
<td>HP0510 is replaced with uPtet02-GFP</td>
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<tr>
<td>hAD17</td>
<td>X47 mdaB::ptetR1; trpA::rpsL-cat</td>
<td><em>promoter-tetR1</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted into HP1277</td>
<td>This work</td>
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<td>hAD18</td>
<td>X47 mdaB::ptetR2; trpA::rpsL-cat</td>
<td><em>promoter-tetR2</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted into HP1277</td>
<td>This work</td>
</tr>
<tr>
<td>hAD19</td>
<td>X47 mdaB::ptetR3; trpA::rpsL-cat</td>
<td><em>promoter-tetR3</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted into HP1277</td>
<td>This work</td>
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<td>hAD20</td>
<td>X47 mdaB::ptetR4; trpA::rpsL-cat</td>
<td><em>promoter-tetR4</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted into HP1277</td>
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<td>hAD21</td>
<td>X47 mdaB::ptetR5; trpA::rpsL-cat</td>
<td><em>promoter-tetR5</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted into HP1277</td>
<td>This work</td>
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<td>hAD22</td>
<td>X47 mdaB::ptetR6; trpA::rpsL-cat</td>
<td><em>promoter-tetR6</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted into HP1277</td>
<td>This work</td>
</tr>
<tr>
<td>hAD23</td>
<td>X47 mdaB::ptetR7; trpA::rpsL-cat</td>
<td><em>promoter-tetR7</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted into HP1277</td>
<td>This work</td>
</tr>
<tr>
<td>hAD24</td>
<td>X47 mdaB::ptetR1; gltDH::rpsL-cat</td>
<td><em>promoter-tetR1</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD25</td>
<td>X47 mdaB::ptetR2; gltDH::rpsL-cat</td>
<td><em>promoter-tetR2</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted between HP0379 and HP380</td>
<td>This work</td>
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<td>hAD26</td>
<td>X47 mdaB::ptetR3; gltDH::rpsL-cat</td>
<td><em>promoter-tetR3</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD27</td>
<td>X47 mdaB::ptetR4; gltDH::rpsL-cat</td>
<td><em>promoter-tetR4</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD28</td>
<td>X47 mdaB::ptetR5; gltDH::rpsL-cat</td>
<td><em>promoter-tetR5</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted between HP0379 and HP380</td>
<td>This work</td>
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<td>hAD29</td>
<td>X47 mdaB::ptetR6; gltDH::rpsL-cat</td>
<td><em>promoter-tetR6</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD30</td>
<td>X47 mdaB::ptetR7; gltDH::rpsL-cat</td>
<td><em>promoter-tetR7</em> inserted between HP630 and HP631; <em>rpsL-cat</em> inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>AD#</td>
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<tr>
<td>hAD31</td>
<td>X47 mdaB::ptetR1; dapB::rpsL-cat</td>
<td><em>promoter-tetR1</em> inserted between HP630 and HP631; HP0510 is replaced with <em>rpsL-cat</em></td>
<td>This work</td>
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<tr>
<td>hAD32</td>
<td>X47 mdaB::ptetR2; dapB::rpsL-cat</td>
<td><em>promoter-tetR2</em> inserted between HP630 and HP631; HP0510 is replaced with <em>rpsL-cat</em></td>
<td>This work</td>
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<tr>
<td>hAD33</td>
<td>X47 mdaB::ptetR3; dapB::rpsL-cat</td>
<td><em>promoter-tetR3</em> inserted between HP630 and HP631; HP0510 is replaced with <em>rpsL-cat</em></td>
<td>This work</td>
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<tr>
<td>hAD34</td>
<td>X47 mdaB::ptetR4; dapB::rpsL-cat</td>
<td><em>promoter-tetR4</em> inserted between HP630 and HP631; HP0510 is replaced with <em>rpsL-cat</em></td>
<td>This work</td>
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<tr>
<td>hAD35</td>
<td>X47 mdaB::ptetR5; dapB::rpsL-cat</td>
<td><em>promoter-tetR5</em> inserted between HP630 and HP631; HP0510 is replaced with <em>rpsL-cat</em></td>
<td>This work</td>
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<tr>
<td>hAD36</td>
<td>X47 mdaB::ptetR6; dapB::rpsL-cat</td>
<td><em>promoter-tetR6</em> inserted between HP630 and HP631; HP0510 is replaced with <em>rpsL-cat</em></td>
<td>This work</td>
</tr>
<tr>
<td>hAD37</td>
<td>X47 mdaB::ptetR7; dapB::rpsL-cat</td>
<td><em>promoter-tetR7</em> inserted between HP630 and HP631; HP0510 is replaced with <em>rpsL-cat</em></td>
<td>This work</td>
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<tr>
<td>hAD38-</td>
<td>X47 mdaB::ptetR(1-6);</td>
<td><em>promoter-tetR</em> inserted between HP630 and HP631; <em>uPtetO-GFP</em> inserted at recipient locus (refer to Table 3-4)</td>
<td>This work</td>
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<tr>
<td>hAD73</td>
<td>(trpA/gltDH/dapB)::uPtetO(1-3)-GFP (SET)</td>
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<tr>
<td>hAD74</td>
<td>X 47 HP0421::rpsL-cat-1</td>
<td><em>rpsL-cat</em> replacement of majority of HP0421</td>
<td>This work</td>
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<tr>
<td>hAD75</td>
<td>X 47 ΔHP0421</td>
<td>HP0421 is completely removed from genome</td>
<td>This work</td>
</tr>
<tr>
<td>hAD76</td>
<td>X 47 ΔHP0421; trpA::rpsL-cat</td>
<td>HP0421 is completely removed from genome; <em>rpsL-cat</em> inserted into HP1277</td>
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<tr>
<td>hAD77</td>
<td>X 47 ΔHP0421; gltDH::rpsL-cat</td>
<td>HP0421 is completely removed from genome; <em>rpsL-cat</em> inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD78</td>
<td>X 47 ΔHP0421; dapB::rpsL-cat</td>
<td>HP0421 is completely removed from genome; HP0510 is replaced with <em>rpsL-cat</em></td>
<td>This work</td>
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<tr>
<td>hAD79</td>
<td>X 47 ΔHP0421; trpA::uPtetO1-CGT</td>
<td>HP0421 is completely removed from genome; <em>uPtetO1-CGT</em> inserted into HP1277</td>
<td>This work</td>
</tr>
<tr>
<td>hAD80</td>
<td>X 47 ΔHP0421; trpA::uPtetO2-CGT</td>
<td>HP0421 is completely removed from genome; <em>uPtetO2-CGT</em> inserted into HP1277</td>
<td>This work</td>
</tr>
<tr>
<td>AD#</td>
<td>Strain name</td>
<td>Description</td>
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<tr>
<td>hAD81</td>
<td>X47 ΔHP0421; gltDH::uPtetO1-CGT</td>
<td>uPtetO1-CGT inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD82</td>
<td>X47 ΔHP0421; gltDH::uPtetO2-CGT</td>
<td>uPtetO2-CGT inserted between HP0379 and HP380</td>
<td>This work</td>
</tr>
<tr>
<td>hAD83</td>
<td>X47 ΔHP0421; dapB::uPtetO1-CGT</td>
<td>HP0421 is completely removed from genome; HP0510 replaced with uPtetO1-CGT</td>
<td>This work</td>
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<tr>
<td>hAD84</td>
<td>X47 ΔHP0421; dapB::uPtetO2-CGT</td>
<td>HP0421 is completely removed from genome; HP0510 replaced with uPtetO1-CGT</td>
<td>This work</td>
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<tr>
<td>hAD85</td>
<td>X47 trpA::uPtetO1-CGT</td>
<td>uPtetO1-CGT inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD86</td>
<td>X47 trpA::uPtetO2-CGT</td>
<td>uPtetO2-CGT inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD87</td>
<td>X47 trpA::uPtetO3-CGT</td>
<td>uPtetO3-CGT inserted into HP1277</td>
<td>This work</td>
</tr>
<tr>
<td>hAD88</td>
<td>X47 gltDH::uPtetO1-CGT</td>
<td>uPtetO1-CGT inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD89</td>
<td>X47 gltDH::uPtetO2-CGT</td>
<td>uPtetO2-CGT inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD90</td>
<td>X47 gltDH::uPtetO3-CGT</td>
<td>uPtetO3-CGT inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD91</td>
<td>X47 mdaB::ptetR(1); trpA::uPtetO1-CGT</td>
<td>promoter-tetR1 inserted between HP630 and HP631; uPtetO1-CGT inserted</td>
<td>This work</td>
</tr>
<tr>
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<td>into HP1277</td>
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</tr>
<tr>
<td>hAD92</td>
<td>X47 mdaB::ptetR(1); trpA::uPtetO2-CGT</td>
<td>promoter-tetR1 inserted between HP630 and HP631; uPtetO2-CGT inserted</td>
<td>This work</td>
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<td>into HP1277</td>
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<tr>
<td>hAD93</td>
<td>X47 mdaB::ptetR(1); gltDH::uPtetO1-CGT</td>
<td>promoter-tetR1 inserted between HP630 and HP631; uPtetO1-CGT inserted</td>
<td>This work</td>
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<td>into HP1277</td>
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<tr>
<td>hAD94</td>
<td>X47 mdaB::ptetR(2); trpA::uPtetO1-CGT</td>
<td>promoter-tetR2 inserted between HP630 and HP631; uPtetO1-CGT inserted</td>
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<td>into HP1277</td>
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<tr>
<td>hAD95</td>
<td>X47 mdaB::ptetR(2); trpA::uPtetO2-CGT</td>
<td>promoter-tetR2 inserted between HP630 and HP631; uPtetO2-CGT inserted</td>
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<td>into HP1277</td>
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<tr>
<td>hAD96</td>
<td>X47 mdaB::ptetR(2); trpA::uPtetO3-CGT</td>
<td>promoter-tetR2 inserted between HP630 and HP631; uPtetO3-CGT inserted</td>
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<tr>
<td></td>
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<td>into HP1277</td>
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<tr>
<td>hAD97</td>
<td>X47 mdaB::ptetR(2); gltDH::uPtetO1-CGT</td>
<td>promoter-tetR2 inserted between HP630 and HP631; uPtetO1-CGT inserted</td>
<td>This work</td>
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<tr>
<td></td>
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<td>into HP0379 and HP380</td>
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<td>AD#</td>
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<td>hAD98</td>
<td>X47 mdaB::ptetR(2); gltDH::uPtetO2-CGT</td>
<td>promoter-tetR2 inserted between HP630 and HP631; uPtetO2-CGT inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD99</td>
<td>X47 mdaB::ptetR(2); gltDH::uPtetO3-CGT</td>
<td>promoter-tetR2 inserted between HP630 and HP631; uPtetO3-CGT inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD100</td>
<td>X47 mdaB::ptetR(4); trpA::uPtetO1-CGT</td>
<td>promoter-tetR4 inserted between HP630 and HP631; uPtetO1-CGT inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD101</td>
<td>X47 mdaB::ptetR(4); trpA::uPtetO2-CGT</td>
<td>promoter-tetR4 inserted between HP630 and HP631; uPtetO2-CGT inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD102</td>
<td>X47 mdaB::ptetR(4); trpA::uPtetO3-CGT</td>
<td>promoter-tetR4 inserted between HP630 and HP631; uPtetO3-CGT inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD103</td>
<td>X47 mdaB::ptetR(4); gltDH::uPtetO1-CGT</td>
<td>promoter-tetR4 inserted between HP630 and HP631; uPtetO1-CGT inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD104</td>
<td>X47 mdaB::ptetR(4); gltDH::uPtetO2-CGT</td>
<td>promoter-tetR4 inserted between HP630 and HP631; uPtetO2-CGT inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD105</td>
<td>X47 mdaB::ptetR(4); gltDH::uPtetO3-CGT</td>
<td>promoter-tetR4 inserted between HP630 and HP631; uPtetO3-CGT inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD106</td>
<td>X47 mdaB::ptetR(6); trpA::uPtetO1-CGT</td>
<td>promoter-tetR6 inserted between HP630 and HP631; uPtetO1-CGT inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD107</td>
<td>X47 mdaB::ptetR(6); trpA::uPtetO2-CGT</td>
<td>promoter-tetR6 inserted between HP630 and HP631; uPtetO2-CGT inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD108</td>
<td>X47 mdaB::ptetR(6); gltDH::uPtetO1-CGT</td>
<td>promoter-tetR6 inserted between HP630 and HP631; uPtetO1-CGT inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD109</td>
<td>X47 mdaB::ptetR(6); gltDH::uPtetO2-CGT</td>
<td>promoter-tetR6 inserted between HP630 and HP631; uPtetO2-CGT inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD110</td>
<td>X47 HP0421::rpsL-cat-2</td>
<td>rpsL-cat replaces majority of HP0421</td>
<td>This work</td>
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<tr>
<td>hAD111</td>
<td>X47 HP0421::rpsL-cat-3</td>
<td>Complete replacement of HP0421 with rpsL-cat</td>
<td>This work</td>
</tr>
<tr>
<td>hAD112</td>
<td>X47 trpA::uPtetO1-CGT, HP0421::rpsL-cat-2</td>
<td>uPtetO1-CGT inserted into HP1277, rpsL-cat replaces majority of HP0421</td>
<td>This work</td>
</tr>
<tr>
<td>AD#</td>
<td>Strain name</td>
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<tr>
<td>hAD113</td>
<td>X47 trpA::uPtetO1-CGT; HP0421::rpsL-cat-3</td>
<td>uPtetO1-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
<td>This work</td>
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<tr>
<td>hAD114</td>
<td>X47 trpA::uPtetO2-CGT; HP0421::rpsL-cat-2</td>
<td>uPtetO2-CGT inserted into HP1277, rpsL-cat replaces majority of HP0421</td>
<td>This work</td>
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<tr>
<td>hAD115</td>
<td>X47 trpA::uPtetO2-CGT; HP0421::rpsL-cat-3</td>
<td>uPtetO2-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
<td>This work</td>
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<tr>
<td>hAD116</td>
<td>X47 trpA::uPtetO3-CGT; HP0421::rpsL-cat-3</td>
<td>uPtetO3-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
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<tr>
<td>hAD117</td>
<td>X47 gltDH::uPtetO1-CGT; HP0421::rpsL-cat-2</td>
<td>uPtetO1-CGT inserted between HP0379 and HP380, rpsL-cat replaces majority of HP0421</td>
<td>This work</td>
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<tr>
<td>hAD118</td>
<td>X47 gltDH::uPtetO1-CGT; HP0421::rpsL-cat-3</td>
<td>uPtetO1-CGT inserted between HP0379 and HP380, complete replacement of HP0421 with rpsL-cat</td>
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<tr>
<td>hAD119</td>
<td>X47 gltDH::uPtetO2-CGT; HP0421::rpsL-cat-2</td>
<td>uPtetO2-CGT inserted between HP0379 and HP380, rpsL-cat replaces majority of HP0421</td>
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<tr>
<td>hAD120</td>
<td>X47 gltDH::uPtetO2-CGT; HP0421::rpsL-cat-3</td>
<td>uPtetO2-CGT inserted between HP0379 and HP380, complete replacement of HP0421 with rpsL-cat</td>
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<td>hAD121</td>
<td>X47 gltDH::uPtetO3-CGT; HP0421::rpsL-cat-3</td>
<td>uPtetO3-CGT inserted between HP0379 and HP380, complete replacement of HP0421 with rpsL-cat</td>
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<tr>
<td>hAD122</td>
<td>X47 mdaB::ptetR(1); trpA::uPtetO1-CGT; HP0421::rpsL-cat-2</td>
<td>promoter-tetR1 inserted between HP630 and HP631; uPtetO1-CGT inserted into HP1277, rpsL-cat replaces majority of HP0421</td>
<td>This work</td>
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<tr>
<td>hAD123</td>
<td>X47 mdaB::ptetR(1); trpA::uPtetO1-CGT; HP0421::rpsL-cat-2</td>
<td>promoter-tetR1 inserted between HP630 and HP631; uPtetO1-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
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<tr>
<td>hAD124</td>
<td>X47 mdaB::ptetR(1); trpA::uPtetO2-CGT; HP0421::rpsL-cat-2</td>
<td>promoter-tetR1 inserted between HP630 and HP631; uPtetO2-CGT inserted into HP1277, rpsL-cat replaces majority of HP0421</td>
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<td>hAD125</td>
<td>X47 mdaB::ptetR(1); trpA::uPtetO2-CGT; HP0421::rpsL-cat-2</td>
<td>promoter-tetR1 inserted between HP630 and HP631; uPtetO2-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
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<tr>
<td>hAD126</td>
<td>X47 mdaB::ptetR(1); gltDH::uPtetO1-CGT; HP0421::rpsL-cat-2</td>
<td>promoter-tetR1 inserted between HP630 and HP631; uPtetO1-CGT inserted between HP0379 and HP380, rpsL-cat replaces majority of HP0421</td>
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<td>hAD127</td>
<td>X47 mdaB::ptetR(1); gltDH::uPtetO1-CGT; HP0421::rpsL-cat-3</td>
<td>promoter-tetR1 inserted between HP630 and HP631; uPtetO1-CGT inserted between HP0379 and HP380, complete replacement of HP0421 with rpsL-cat</td>
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Table 3-2  Strains used in this study, continued

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<td>trpA::uPtetO1-CGT; promoter-tetR2 inserted between HP630 and HP631; uPtetO1-CGT inserted into HP1277, rpsL-cat replaces majority of HP0421</td>
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<td>X47  mdaB::ptetR(2); HP0421::rpsL-cat-3</td>
<td>trpA::uPtetO1-CGT; promoter-tetR2 inserted between HP630 and HP631; uPtetO1-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
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<td>hAD130</td>
<td>X47  mdaB::ptetR(2); HP0421::rpsL-cat-2</td>
<td>trpA::uPtetO2-CGT; promoter-tetR2 inserted between HP630 and HP631; uPtetO2-CGT inserted into HP1277, rpsL-cat replaces majority of HP0421</td>
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<td>X47  mdaB::ptetR(2); HP0421::rpsL-cat-3</td>
<td>trpA::uPtetO2-CGT; promoter-tetR2 inserted between HP630 and HP631; uPtetO2-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
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<td>X47  mdaB::ptetR(2); HP0421::rpsL-cat-3</td>
<td>trpA::uPtetO3-CGT; promoter-tetR2 inserted between HP630 and HP631; uPtetO3-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
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<td>hAD133</td>
<td>X47  mdaB::ptetR(2); gltDH::uPtetO1-CGT; HP0421::rpsL-cat-2</td>
<td>trpA::uPtetO1-CGT; promoter-tetR2 inserted between HP630 and HP631; uPtetO1-CGT inserted between HP0379 and HP380, rpsL-cat replaces majority of HP0421</td>
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<td>X47  mdaB::ptetR(2); gltDH::uPtetO1-CGT; HP0421::rpsL-cat-3</td>
<td>trpA::uPtetO1-CGT; promoter-tetR2 inserted between HP630 and HP631; uPtetO1-CGT inserted between HP0379 and HP380, complete replacement of HP0421 with rpsL-cat</td>
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<td>hAD135</td>
<td>X47  mdaB::ptetR(2); gltDH::uPtetO2-CGT; HP0421::rpsL-cat-2</td>
<td>trpA::uPtetO2-CGT; promoter-tetR2 inserted between HP630 and HP631; uPtetO2-CGT inserted between HP0379 and HP380, rpsL-cat replaces majority of HP0421</td>
<td>This work</td>
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<tr>
<td>hAD136</td>
<td>X47  mdaB::ptetR(2); gltDH::uPtetO3-CGT; HP0421::rpsL-cat-3</td>
<td>trpA::uPtetO3-CGT; promoter-tetR2 inserted between HP630 and HP631; uPtetO3-CGT inserted between HP0379 and HP380, complete replacement of HP0421 with rpsL-cat</td>
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<tr>
<td>hAD137</td>
<td>X47  mdaB::ptetR(4); HP0421::rpsL-cat-2</td>
<td>trpA::uPtetO1-CGT; promoter-tetR4 inserted between HP630 and HP631; uPtetO1-CGT inserted into HP1277, rpsL-cat replaces majority of HP0421</td>
<td>This work</td>
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<tr>
<td>hAD138</td>
<td>X47  mdaB::ptetR(4); HP0421::rpsL-cat-3</td>
<td>trpA::uPtetO1-CGT; promoter-tetR4 inserted between HP630 and HP631; uPtetO1-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
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<td>X47  mdaB::ptetR(4); HP0421::rpsL-cat-2</td>
<td>trpA::uPtetO2-CGT; promoter-tetR4 inserted between HP630 and HP631; uPtetO2-CGT inserted into HP1277, rpsL-cat replaces majority of HP0421</td>
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<td>hAD140</td>
<td>X47  mdaB::ptetR(4); HP0421::rpsL-cat-3</td>
<td>trpA::uPtetO2-CGT; promoter-tetR4 inserted between HP630 and HP631; uPtetO2-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
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<td>hAD141</td>
<td>X47  mdaB::ptetR(4); HP0421::rpsL-cat-3</td>
<td>trpA::uPtetO3-CGT; promoter-tetR4 inserted between HP630 and HP631; uPtetO3-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
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<tr>
<td>AD#</td>
<td>Strain name</td>
<td>Description</td>
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<td>X47 mdaB::ptetR; gltDH::uPtetO1-CGT; HP0421::rpsL-cat-2</td>
<td>promoter-tetR4 inserted between HP630 and HP631; uPtetO1-CGT inserted between HP0379 and HP380, rpsL-cat replaces majority of HP0421</td>
<td>This work</td>
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<tr>
<td>hAD143</td>
<td>X47 mdaB::ptetR(4); gltDH::uPtetO2-CGT; HP0421::rpsL-cat-2</td>
<td>promoter-tetR4 inserted between HP630 and HP631; uPtetO2-CGT inserted between HP0379 and HP380, rpsL-cat replaces majority of HP0421</td>
<td>This work</td>
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<tr>
<td>hAD144</td>
<td>X47 mdaB::ptetR(4); gltDH::uPtetO3-CGT; HP0421::rpsL-cat-3</td>
<td>promoter-tetR4 inserted between HP630 and HP631; uPtetO3-CGT inserted between HP0379 and HP380, complete replacement of HP0421 with rpsL-cat</td>
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<tr>
<td>hAD145</td>
<td>X47 mdaB::ptetR(6); trpA::uPtetO1-CGT; HP0421::rpsL-cat-2</td>
<td>promoter-tetR6 inserted between HP630 and HP631; uPtetO1-CGT inserted into HP1277, rpsL-cat replaces majority of HP0421</td>
<td>This work</td>
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<tr>
<td>hAD146</td>
<td>X47 mdaB::ptetR(6); trpA::uPtetO2-CGT; HP0421::rpsL-cat-2</td>
<td>promoter-tetR6 inserted between HP630 and HP631; uPtetO2-CGT inserted into HP1277, rpsL-cat replaces majority of HP0421</td>
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<tr>
<td>hAD147</td>
<td>X47 mdaB::ptetR(6); gltDH::uPtetO1-CGT; HP0421::rpsL-cat-2</td>
<td>promoter-tetR6 inserted between HP630 and HP631; uPtetO1-CGT inserted between HP0379 and HP380, rpsL-cat replaces majority of HP0421</td>
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<tr>
<td>hAD148</td>
<td>X47 mdaB::ptetR(6); gltDH::uPtetO2-CGT; HP0421::rpsL-cat-2</td>
<td>promoter-tetR6 inserted between HP630 and HP631; uPtetO2-CGT inserted between HP0379 and HP380, rpsL-cat replaces majority of HP0421</td>
<td>This work</td>
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<tr>
<td>hAD149</td>
<td>X47 HP0421::rpsL-cat-2, SR</td>
<td>rpsL-cat replaces majority of HP0421, Str^2</td>
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<tr>
<td>hAD150</td>
<td>X47 HP0421::rpsL-cat-3, SR</td>
<td>Complete replacement of HP0421 with rpsL-cat, Str^1</td>
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<tr>
<td>hAD151</td>
<td>X47 trpA::uPtetO1-CGT; HP0421::rpsL-cat-2, SR</td>
<td>uPtetO1-CGT inserted into HP1277, rpsL-cat replaces majority of HP0421, Str^1</td>
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<tr>
<td>hAD152</td>
<td>X47 trpA::uPtetO1-CGT; HP0421::rpsL-cat-3, SR</td>
<td>uPtetO1-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat, Str^1</td>
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<tr>
<td>hAD153</td>
<td>X47 gltDH::uPtetO1-CGT; HP0421::rpsL-cat-2, SR</td>
<td>uPtetO1-CGT inserted between HP0379 and HP380, rpsL-cat replaces majority of HP0421, Str^1</td>
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<tr>
<td>hAD154</td>
<td>X47 gltDH::uPtetO1-CGT; HP0421::rpsL-cat-3, SR</td>
<td>uPtetO1-CGT inserted between HP0379 and HP380, complete replacement of HP0421 with rpsL-cat, Str^1</td>
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<td>hAD155</td>
<td>X47 mdaB::ptetR(1); trpA::uPtetO2-CGT; HP0421::rpsL-cat-2, SR</td>
<td>promoter-tetR1 inserted between HP630 and HP631; uPtetO2-CGT inserted into HP1277, rpsL-cat replaces majority of HP0421, Str^1</td>
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<tr>
<td>hAD156</td>
<td>X47 mdaB::ptetR(1); trpA::uPtetO2-CGT; HP0421::rpsL-cat-3, SR</td>
<td>promoter-tetR1 inserted between HP630 and HP631; uPtetO2-CGT inserted into HP1277, complete replacement of HP0421 with rpsL-cat, Str^1</td>
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<td>X47 CGT-HA (SET)</td>
<td>Series of X47 strains expressing CGT with a C-terminal HA peptide tag (refer to Table 3-5)</td>
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<td>hAD175</td>
<td>X47 ureA::rpsL-cat</td>
<td>ureA and upstream promoter replaced with rpsL-cat</td>
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<td>hAD176</td>
<td>X47 urePtetOI</td>
<td>Two tetO operators located in ureA promoter</td>
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<td>hAD177</td>
<td>X47 urePtetOII</td>
<td>One tetO operator located in ureA promoter</td>
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<tr>
<td>hAD178</td>
<td>X47 urePtetOIII</td>
<td>Three tetO operators located in ureA promoter</td>
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<tr>
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<td>X47 urePtetOIV</td>
<td>Two tetO operators located in ureA promoter</td>
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<td>X47 urePtetOV</td>
<td>One tetO operators located in ureA promoter</td>
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<td>hAD181</td>
<td>X47 mdaB::petR1; ureA::rpsL-cat</td>
<td>promoter-tetR1 inserted between HP630 and HP631; ureA and upstream promoter replaced with rpsL-cat</td>
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<td>hAD182</td>
<td>X47 mdaB::petR2; ureA::rpsL-cat</td>
<td>promoter-tetR2 inserted between HP630 and HP631; ureA and upstream promoter replaced with rpsL-cat</td>
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<td>hAD183</td>
<td>X47 mdaB::petR3; ureA::rpsL-cat</td>
<td>promoter-tetR3 inserted between HP630 and HP631; ureA and upstream promoter replaced with rpsL-cat</td>
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<td>hAD184</td>
<td>X47 mdaB::petR4; ureA::rpsL-cat</td>
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<td>hAD185</td>
<td>X47 mdaB::petR5; ureA::rpsL-cat</td>
<td>promoter-tetR5 inserted between HP630 and HP631; ureA and upstream promoter replaced with rpsL-cat</td>
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<td>hAD186</td>
<td>X47 mdaB::petR6; ureA::rpsL-cat</td>
<td>promoter-tetR6 inserted between HP630 and HP631; ureA and upstream promoter replaced with rpsL-cat</td>
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<td>hAD187</td>
<td>X47 mdaB::petR7; ureA::rpsL-cat</td>
<td>promoter-tetR7 inserted between HP630 and HP631; ureA and upstream promoter replaced with rpsL-cat</td>
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<td>hAD188</td>
<td>X47 mdaB::petR(1-7); urePtetO(I-V) (SET)</td>
<td>promoter-tetR inserted between HP630 and HP631; tetO operators located in ureA promoter (refer to Table 3-7)</td>
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<td>hAD189-</td>
<td>X47 mdaB::petR; urePtetO(1-7)</td>
<td>promoter-tetR inserted between HP630 and HP631; ureA promoter replaced with urePtetO; re-isolated from C57BL/6J mouse</td>
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<td>hAD221</td>
<td>X47 mdaB::petR4; urePtetO-MP clone 1</td>
<td>promoter-tetR inserted between HP630 and HP631; ureA promoter replaced with urePtetO; re-isolated from C57BL/6J mouse</td>
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<td>hAD222</td>
<td>X47 mdaB::petR4; urePtetO-MP clone 2</td>
<td>promoter-tetR inserted between HP630 and HP631; ureA promoter replaced with urePtetO; re-isolated from C57BL/6J mouse</td>
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<td>AD #</td>
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<td>X47 mdaB::ptetR4; urePtetOI-MP clone 4</td>
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<td>X47 mdaB::ptetR4; urePtetOI-MP clone 5</td>
<td><em>promoter-tetR</em> inserted between HP630 and HP631; <em>ureA</em> promoter replaced with <em>urePtetOI</em>; re-isolated from C57BL/6J mouse</td>
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<tr>
<td>hAD227</td>
<td>X47 mdaB::ptetR4; urePtetOI-MP clone 6</td>
<td><em>promoter-tetR</em> inserted between HP630 and HP631; <em>ureA</em> promoter replaced with <em>urePtetOI</em>; re-isolated from C57BL/6J mouse</td>
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Table 3-3  Oligonucleotide primers used in this study continued

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3.2 Development of tet-regulation system in *H. pylori*

3.2.1 Construction of cloning vectors for integration of DNA into the *H. pylori* genome

Three regions in the *H. pylori* chromosome were identified as potential recipient loci for the integration and expression of foreign DNA. X47 strains with gene insertions between HP0379 and HP0380 (*gltDH* locus), within HP1277 (*trpA* locus) and replacement of HP0510 (*dapB* locus) did not display noticeable growth defects and were still able to colonize C57BL/6J mice (private communication from Dr. Ji Kevin Li and Ondek Pty. Ltd.).

Cloning vectors containing two neighbouring regions of the *H. pylori* genome, for targeted homologous recombination, separated by a small multiple cloning site (MCS) were constructed for these three loci in three steps (Figure 3-1, specific example is for pGltDH).

Step 1 involved amplifying two neighbouring regions, arm I and arm II, in the genomic locus of interest using *H. pylori* 26695 genomic DNA as template DNA. Arm I was amplified using primers numbered 1 and 2 and the downstream region, arm II, was amplified using primers numbered 3 and 4. Primers 2 and 3 had long complementary sequences at their 5’ ends that served to introduce several unique restriction sites, generating the MCS. The standard PCR reactions in this step contained DMSO and were performed using *Pwo* polymerase. The PCR products were purified, using a PCR purification kit, and then treated with *DpnI* for two hours at 37 °C to remove genomic template DNA.

Step 2 involved fusing arms I and II together by SOE PCR, the principles of SOE PCR have been described (see section 2.2.2.3), using an equal mixture of arm I and arm II and primers numbered 1 and 4.

Step 3 involved cloning the PCR products from Step 2 into either pBlu-SK-alt or pHSG576. The ligation products were transformed into *E. coli* DH5α. Recombinant clones were screened by colony PCR and confirmed by restriction enzyme mapping, and sequencing.
Chapter 3: Plasmid and strain construction

Figure 3-1  Construction of cloning vector pGltDH (pAD303)
The cloning vector for the \textit{gltDH} locus, pGltDH (Figure 3-1) (Table 3-1, pAD301), was designed to insert foreign DNA in the intergenic region between the HP0379 and HP0380 ORFs. The cloning vector for the \textit{trpA} locus, pTrpA (Figure 3-2 A) (Table 3-1, pAD301), was designed to insert foreign DNA into the center of the HP1277 ORF. For the \textit{dapB} locus, the pHdapB cloning vector (Figure 3-2 B) (Table 3-1, pAD305) was designed to replace the HP0501 ORF in \textit{H. pylori} with foreign DNA sequences.

**3.2.1.1 Construction of pGltDH**

1 kb of the 3’ end of HP0380 was amplified with primers GltDH1 and GltDH2 and 1 kb of HP00379 was amplified with primers GltDH3 and GltDH4 (Figure 3-3 A). The two PCR fragments were joined together by SOE PCR, using an extension time of 60 s for the first 10 cycles, followed by the addition of primers GltDH1 and GltDH4 and an increase of extension time to 120 s. The resulting 2 kb PCR product contained a 36 bp MCS inserted between HP0380 and HP0378 sequences and was flanked on either end by \textit{ClaI} and \textit{SacII} restriction sites (Figure 3-3 D). This 2 kb fragment was cloned into pBlu-SK-alt, using \textit{ClaI} and \textit{SacII} restriction sites, to give pGltDH (Figure 3-1).

**3.2.1.2 Construction of pTrpA**

A similar methodology to the one described in 3.2.1.1 was used to generate pTrpA. Using the same PCR conditions, 1 kb region upstream from the centre of the HP1277 ORF and a 1 kb region downstream of the centre of the HP1277 ORF was amplified using primers TrpA1 and TrpA2, and primers TrpA3 and TrpA4 respectively (Figure 3-3 B). The two PCR fragments were joined together by SOE PCR (Figure 3-3 D) and cloned into pBlu-SK-alt to give pTrpA (Figure 3-2 A).
Figure 3-3  PCR construction of cloning regions for cloning vectors

A. Amplification of arms I and II for pGltDH
   Lane 1 – 4: amplification of arm I
   Specific conditions
   Lane 1: Annealing temperature 50 °C, 5% DMSO
   Lane 2: Annealing temperature 55 °C, 5% DMSO
   Lane 3: Annealing temperature 50 °C
   Lane 4: Annealing temperature 55 °C
   Lane 5 – 8: amplification of arm II
   Specific conditions
   Lane 5: Annealing temperature 50 °C, 5% DMSO
   Lane 6: Annealing temperature 55 °C, 5% DMSO
   Lane 7: Annealing temperature 50 °C
   Lane 8: Annealing temperature 55 °C

B. Amplification of arms I and II for pTrpA
   Lane 1 – 4: amplification of arm I
   Specific conditions
   Lane 1: Annealing temperature 50 °C, 5% DMSO
   Lane 2: Annealing temperature 55 °C, 5% DMSO
   Lane 3: Annealing temperature 50 °C
   Lane 4: Annealing temperature 55 °C
   Lane 5 – 6: amplification of arm II
   Specific conditions
   Lane 5: Annealing temperature 50 °C, 5% DMSO
   Lane 6: Annealing temperature 55 °C, 5% DMSO
C. Amplification of arms I and II for pHdapB
   Lane 1 – 6: amplification of arm I
   Specific conditions
   Lane 1: Annealing temperature 45 °C, 5% DMSO
   Lane 2: Annealing temperature 50 °C, 5% DMSO
   Lane 3: Annealing temperature 55 °C, 5% DMSO
   Lane 4: Annealing temperature 45 °C
   Lane 5: Annealing temperature 50 °C
   Lane 6: Annealing temperature 55 °C
   Lane 7 – 12: amplification of arm II
   Specific conditions
   Lane 7: Annealing temperature 45 °C, 5% DMSO
   Lane 8: Annealing temperature 50 °C, 5% DMSO
   Lane 9: Annealing temperature 55 °C, 5% DMSO
   Lane 10: Annealing temperature 45 °C
   Lane 11: Annealing temperature 50 °C
   Lane 12: Annealing temperature 55 °C

D. SOE PCR products from joining arms I and II
   Lane 1: arm I + arm II for pGltDH
   Lane 2: arm I + arm II for pTrpA

E. SOE PCR products from joining arms I and II
   Lane 1: arm I + arm II pHdapB

### 3.2.1.3 Construction of pHdapB

A slightly different strategy was used in the construction of pHdapB. Two 600 bp regions flanking the HP0510 ORF were amplified using primers DapB1 and DapB2, and primers DapB3 and DapB4 (Figure 3-3 C). The two PCR fragments were joined together by SOE PCR, using an extension time of 40 s for the first 10 cycles, followed by the addition of primers DapB5 and DapB6 and an increase of extension time to 90 s (Figure 3-3 E). The resulting 1.2 kb PCR product was composed of the sequences upstream and downstream of the HP0510 ORF separated by a small MCS and was flanked on either end by HindIII and EcoRI restriction sites. The final product was cloned into pHSG576, using HindIII and EcoRI restriction sites, to create pHdapB (Figure 3-2 B).

### 3.2.1.4 Characterization of cloning vectors

Restriction enzyme digestion of plasmids isolated from E. coli transformants identified clones with correct insertion of the SOE PCR product into the recipient vectors. pGltDH and pTrpA vectors were digested with XbaI, BglII, EcoRI and BamHI to confirm the
plasmid size and the integrity of the MCS (Figure 3-4 A). All the analysed clones had
the correct plasmid size and all the restriction sites of the MCS were intact, although the
BgII digest was incomplete for pGltDH and poor for pTrpA. This was found to be due
to the stock of BgIII enzyme used. A repeat digestion with a new stock of BgIII showed
complete digestion of the vectors. pHdapB was digested with HindIII to linearize
the plasmid, or with both HindIII and EcoRI to excise the insert (Figure 3-4 B). All
screened clones had the correct plasmid size and contained the flanking dapB region
insert. Plasmid isolated from a colony produced by transformation of E. coli DH5α with
a control ligation reaction (no insert) was used as a negative control.

### 3.2.1.5 Construction of plasmids for generating recipient X47 strains

A set of plasmids containing rpsL-cat was constructed so that the counterselection
method (see section 2.2.3.3) could be used to generate markerless mutations in the
gltDH, trpA, and dapB loci. Plasmid pENT-RC was digested using BamHI to excise the
rpsL-cat counterselection cassette. The 1.3 kb fragment was gel purified and cloned into
the BamHI site of pGltDH, pTrpA, and pHdapB to generate pGltDH-RCAT, pTrpA-
RCAT, and pHdapB-RCAT respectively (Figure 3-5 Specific example for pGltDH-
RCAT and Figure 3-6) (Table 3-1, pAD302, pAD304, pAD306). Correct insertion of
rpsL-cat was confirmed by restriction enzyme digest of plasmid isolated form E. coli
transformants. For pGltDH-RCAT and pTrpA-RCAT, plasmids were digested with Clal
which cut once in the recipient plasmid backbone and once in the rpsL-cat cassette to
release either a 1.2 kb or 2.4 kb insert for pGltDH-RCAT (Figure 3-7 A) and a 1 kb or
2.3 kb insert for pTrpA-RCAT (Figure 3-7 B) depending on the orientation of the rpsL-
cat cassette in these vectors. For pGltDH-RCAT, the rpsL-cat insert was in the same
orientation for all four clones, each releasing the small 1.1 kb insert upon treatment with
ClaI, while for pTrpA-RCAT, clones 1 and 2 contained the rpsL-cat cassette in opposite
orientations and clones c3 and c4 contained multiple copies of the rpsL-cat cassette as
indicated by the additional 1.4 kb band released upon digestion with ClaI. For pHdapB-
RCAT, plasmids were digested with BamHI to excise a band of 1.4 kb corresponding to
the rpsL-cat insert (Figure 3-7 C). Treatment of pHdapB-RCAT plasmids with XhoI
confirmed the size of each plasmid clone and confirmed that only one copy of rpsL-cat
was present.
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Figure 3-4  Diagnostic digest of cloning vectors

A. Restriction analysis of plasmid isolated from *E. coli* transformants for pGltDH and pTrpA
   Lane 1 – 5: pGltDH clone 1
   Specific conditions
   Lane 1: Uncut
   Lane 2: *XhoI* digest (5 kb)
   Lane 3: *BgII* digest - incomplete
   Lane 4: *EcoRI* digest
   Lane 5: *BamHI* digest
   Lane 6 – 10: pGltDH clone 2
   Lane 11 – 15: pTrpA clone 1
   Lane 12: *XhoI* digest (4.8 kb)
   Lane 16 – 20: pTrpA clone 2

B. Restriction analysis of plasmid isolated from *E. coli* transformants for pHdapB
   Lane 1 – 3: vector control – no insert in ligation reaction
   Specific conditions
   Lane 1: Uncut
   Lane 2: *HindIII* digest (4.7 kb)
   Lane 3: *HindIII* and *EcoRI* double digest (1.2 kb and 3.5 kb)
   Lane 4 – 6: pHdapB clone 1
   Lane 7 – 9: pHdapB clone 2
   Lane 10 – 12: pHdapB clone 3
Figure 3-5 Construction of recipient generating plasmid pGltDH-RCAT (pAD304)
3.2.1.6 Construction of *H. pylori* recipient strains at the *gltDH*, *trpA* and *dapB* loci

In order to generate markerless mutations at a chosen recipient locus, recipient strains harbouring *rpsL-cat* at the target locus were required. Recipient strains were made by natural transformation of strain X47 using *Sac*II linearized pGltDH-RCAT, pTrpA-RCAT and pHdapB-RCAT plasmids. Chromosomal DNA of X47 strains transformed at these loci was screened for correct insertion or replacement of the *rpsL-cat* cassette by colony PCR (method 2.2.2.3) using the Exte PCR program, an annealing temperature of 55 °C and primers GltDH1 and GltDH4, TrpA1 and TrpA4, and DapB1 and DapB4 for the *gltDH*, *trpA* and *dapB* loci respectively. Recombinant *H. pylori* X47 strains harbouring *rpsL-cat* at these loci are denoted as *gltDH::rpsL-cat*, *trpA::rpsL-cat*, and *dapB::rpsL-cat*. For *H. pylori* X47 *gltDH::rpsL-cat* and *H. pylori* X47 *trpA::rpsL-cat* mutants, PCR analysis of Cm′ transformants amplified PCR products which were 1.4 kb larger than the PCR products amplified from the wild-type strain, this difference in size corresponded to insertion of the *rpsL-cat* cassette into the *gltDH* and *trpA* loci (Figure 3-8 A). For *H. pylori* X47 *dapB::rpsL-cat* mutants, PCR analysis of Cm′ transformants amplified PCR products that were 700 bp larger than the PCR products amplified from the wild-type strain, corresponding to replacement of the *dapB* gene (765 bp) with the *rpsL-cat* cassette (1.43 kb) (Figure 3-8 B).
Figure 3-7  Diagnostic digest of *rpsL-cat* vectors

A. Restriction analysis of plasmid isolated from *E. coli* transformants for pGltDH-RCAT
Lane 1 – 2: pGltDH-RCAT clone 1
   Lane 1: Uncut
   Lane 2: *ClaI* digest (1.2 kb and 5.2 kb or 2.4 kb and 4 kb)
Lane 3 – 4: pGltDH-RCAT clone 2
Lane 5 – 6: pGltDH-RCAT clone 3
Lane 7 – 8: pGltDH-RCAT clone 4

B. Restriction analysis of plasmid isolated from *E. coli* transformants for pTrpA-RCAT
Lane 1 – 2: pTrpA-RCAT clone 1
   Lane 1: Uncut
   Lane 2: *ClaI* digest (1 kb and 5.2 kb or 2.3 kb and 3.9 kb)
Lane 3 – 4: pTrpA-RCAT clone 2
Lane 5 – 6: pTrpA-RCAT clone 3
Lane 7 – 8: pTrpA-RCAT clone 4

C. Restriction analysis of plasmid isolated from *E. coli* transformants for pHdapB-RCAT
Lane 1 – 2: vector control – no insert in ligation reaction
   Specific conditions
   Lane 1: Uncut
   Lane 2: *BamHI* digest
Lane 3 – 4: pHdapB-RCAT clone 1
   Lane 3: Uncut
   Lane 4: *BamHI* digest (4.7 kb and 1.4 kb)
Lane 5 – 6: pHdapB-RCAT clone 2
Lane 7 – 8: pHdapB-RCAT clone 3
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Figure 3-8  PCR analysis of \textit{gltDH}, \textit{trpA} and \textit{dapB} loci of recipient strains

A. PCR analysis using primers GltDH1 and GltDH4 on genomic DNA isolated from
   Lane 1 – 3: \textit{H. pylori} X47 \textit{gltDH::rpsL-cat} mutants
   Lane 4: \textit{H. pylori} X47 wild-type
   Primers TrpA1 and TrpA4 were used to analyse genomic DNA isolated from
   Lane 5 – 7: \textit{H. pylori} X47 \textit{trpA::rpsL-cat} mutants
   Lane 8: \textit{H. pylori} X47 wild-type

B. PCR analysis using primers DapB1 and DapB4 on genomic DNA isolated from
   Lane 1 – 5: \textit{H. pylori} X47 \textit{dapB::rpsL-cat} mutants
   Lane 6: \textit{H. pylori} X47 wild-type
3.2.2 Construction of *H. pylori* strains expressing *tet* repressors

Four different *H. pylori* promoters were selected to drive expression of TetRs in *H. pylori*. The *amiE*, *flaA*, and wild-type and mutated core *ureA* promoters, were used to generate eight different promoter-*tetR* fusions (*ptetR1-*8*) (Figure 3-9).

![Figure 3-9 Schematic diagram of promoter-*tetR* constructs, *ptetR*](image)

### 3.2.2.1 Generation of *H. pylori* *amiE* promoter-*tetR* fusion constructs: *ptetR1* and *ptetR2*

To generate constructs *ptetR1* and *ptetR2*, (Figure 3-10 A), the *amiE* promoter region, 261 bp sequence of DNA upstream of the *amiE* (HP0294) start codon, was amplified from 26695 genomic DNA using primers tet1 and tet2, using *Pwo* polymerase and an annealing temperature of 55 °C (Figure 3-11 A). The sequences of *tetR* and *revTetR* were amplified from pWH1925 BD and pWH1925 r2 respectively (Table 3-1) with primers tet3 and tet9, using *Pwo* polymerase, 5% DMSO and an annealing temperature of 55 °C (Figure 3-11 A). The amplified *amiE* promoter region, denoted P*amiE*, and *tet* repressor genes were joined together by SOE PCR. Primers tet4 and tet10 were used to amplify the fusion PCR product and introduce flanking *SalI* and *BamHI* sites (Figure 3-11 B).
3.2.2.2 Generation of *H. pylori* flaA promoter-tetR fusion constructs: ptetR3 and ptetR4

A similar strategy to the one described in 3.2.2.1 was used to generate ptetR3 and ptetR4. The *flaA* promoter region, 162 bp of DNA upstream of the *flaA* (HP0601) start codon, was amplified using primers tet5 and tet6 (Figure 3-11 A). The sequences of *tetR* and *revtetR* were amplified using primers tet7 and tet9. SOE PCR using primers tet8 and tet10 was done to join the *flaA* promoter, denoted P_{flaA}, to each *tet* repressor gene and introduce flanking *SalI* and *BamHI* sites (Figure 3-11 B).

3.2.2.3 Generation if *H. pylori* mutated core ureA promoter-tetR fusion constructs: ptetR5 and ptetR6

A different strategy was used to generate the mutated core *ureA* promoter-tetR fusions, ptetR5-8. As the core promoter is small, region -50 to 0 of the promoter and the 5′ UTR (108 bp total), three sequential rounds of standard PCR, using three long forward primers and one short reverse primer, was done to fuse the core *ureA* promoter to the *tet* repressor genes in a step wise manner (Figure 3-10 B). Step 1: *tet* repressor genes were amplified using long forward primer tet11 with reverse primer tet9. The PCR product was purified and used as DNA template in Step 2, using forward primers tet12 with primer tet9. The amplified PCR product was purified and used as DNA template in the final step, Step 3, using forward primer tet14 with reverse primer tet10 to complete the *ureA* core promoter-tetR fusion construct and introduce flanking *SalI* and *BamHI* sites (Figure 3-11 B). Primer tet12 introduced a C to T mutation in the -10 region (taCaat) of the *ureA* promoter. The mutated core *ureA* promoter is denoted as P_{taTaat}. All reactions contained 5% DMSO and were performed using *Pwo* polymerase and an annealing temperature of 55 °C.

3.2.2.4 Generation of *H. pylori* core ureA promoter-tetR fusion constructs: ptetR7 and ptetR8

The same method described above (3.2.2.3) was used to generate ptetR7 and ptetR8 with the exception that in Step 2 primer tet13 was used instead of primer tet12, preserving the wild-type -10 region of the *ureA* promoter. The wild-type core *ureA* promoter is denoted as P_{taCaat}.
Figure 3-10  Construction of promoter-tetR, \( ptetR \)
**Figure 3-10  Construction of promoter-tetR, \( ptetR \) (adjacent page)**

A. Construction of \( ptetR(1-4) \) by SOE PCR. Specific example is for \( ptetR2 \).

B. Construction of \( ptetR(5-8) \) by three consecutive rounds of standard PCR reactions. Specific example is for \( ptetR6 \).

**Figure 3-11  PCR construction of \( ptetR \)**

A. PCR amplification of components for \( ptetR1-4 \) constructs. Lane 1: \( amiE \) promoter region, Lane 2: \( flaA \) promoter region. Lane 3 \( tetR \) and Lane 4: \( revtetR \) for fusion to the \( amiE \) promoter. Lane 5: \( tetR \) and Lane 6: \( revtetR \) for fusion to the \( flaA \) promoter.

B. PCR amplification of final \( ptetR \) constructs. Lane 1: \( P_{amiE} \)-revtetR, Lane 2: \( P_{amiE}-tetR \), Lane 3: \( P_{flaA}-revtetR \), Lane 4: \( P_{taTaat}-revtetR \), Lane 5: \( P_{taTaat}-tetR \), Lane 6: \( P_{taCaat}-revtetR \), Lane 7: \( P_{taCaat}-tetR \) and Lane 8: \( P_{flaA}-tetR \)
3.2.2.5  Transformation of \textit{ptetR}(1-7) constructs into \textit{H. pylori}

All \textit{ptetR} constructs were cloned into vector pHRdx (Croxen et al., 2006; Takeshita et al., 1987) using \textit{Bam}HI and \textit{Sal}I restriction sites to generate pHRdx-\textit{ptetR}(1-8). These plasmids were used to transform the recipient strain \textit{H. pylori} X47 \textit{rdxA::rpsL-cat} to replace the counterselection cassette in the \textit{rdxA} locus with the \textit{ptetR} constructs. However no transformants harbouring the \textit{ptetR} construct could be obtained after several attempts. All colonies isolated on streptomycin containing plates either contained the \textit{rpsL-cat} cassette and were Cm\textsuperscript{r} or had reverted to the wild-type phenotype. To address this problem an alternative recipient locus, the region between HP0630 and HP0631 (\textit{mdaB} locus) (Dailidiene et al., 2006), was evaluated for \textit{ptetR} integration. Insertions in this region had been recently identified as neutral with regards to colonization (Ondek Pty. Ltd., unpublished data). Primers mbtetF and mbtetR were used at an annealing temperature of 55 °C to amplify the \textit{ptetR} constructs from pHRdx-\textit{ptetR}(1-7) plasmid templates and introduce flanking \textit{SbfI} and \textit{EcoRI} cut sites. Amplified \textit{ptetR} constructs were cloned into the pMdaB cloning vector to generate plasmids pMdaB-\textit{ptetR}(1-7) (Table 3-1, pAD307-pAD313), where \textit{ptetR}(1-7) is flanked by 1 kb of DNA sequences encoding the HP0630 and HP0631 genomic region (Figure 3-12). Correct incorporation of the \textit{ptetR} constructs was confirmed by restriction enzyme digest using \textit{Bam}HI which cut twice in the recipient plasmid backbone to release a 1.7 kb insert containing the \textit{ptetR} construct (Figure 3-13 A. Specific example for pMdaB-\textit{ptetR}1). Natural transformation of \textit{H. pylori} recipient strain X47 \textit{mdaB::rpsL-cat} with \textit{Sac}II linearized pMdaB-\textit{ptetR}(1-7) plasmid constructs was performed to generate strains X47 \textit{mdaB::ptetR}(1-7) (Table 3-2, hAD1-hAD7). Chromosomal DNA of the resulting Str\textsuperscript{r} Cm\textsuperscript{s} X47 \textit{mdaB::ptetR}(1-7) strains was screened for correct replacement of the \textit{rpsL-cat} cassette with a \textit{ptetR} construct by colony PCR (method 2.2.2.3) using the Exte PCR program, primers MdaBF1 and MdaBR2 and an annealing temperature of 55 °C (Figure 3-13 B).
Figure 3-12  Construction of pMdaB-ptetR(1-7) (pAD307-pAD313)
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Figure 3-13 Diagnostic digest of pMdaB-ptetR plasmids and PCR analysis of *H. pylori* X47 mdaB::ptetR transformants

A. Restriction analysis of plasmid isolated from *E. coli* transformants for pMdaB-ptetR, specific example is of pMdaB-ptetR1
Lane 1 – 2: vector control – no insert in ligation reaction
Specific conditions
Lane 1: Uncut
Lane 2: *BamHI*
Lane 3 – 4: pMdaB-ptetR1 clone 1
Lane 3: Uncut
Lane 4: *BamHI* digest (4.3 kb and 1.7 kb)
Lane 5 – 6: pMdaB-ptetR1 clone 2
Lane 7 – 8: pMdaB-ptetR1 clone 3

B. PCR analysis using primers MdaBF1 and MdaBR2 on genomic DNA isolated from Lane 1-10: *H. pylori* X47 mdaB::ptetR4 strains, Lane 11: *H. pylori* X47 mdaB::rpsL-cat recipient (3.6 kb) and Lane 12: *H. pylori* X47 wild-type (2.2 kb). Lanes 3, 5-7, 9 and 10 are positive for the *ptetR* construct. Similar analysis was done to screen the other *H. pylori* X47 mdaB::ptetR strains in the set.
3.2.3 Construction of tet-responsive promoter \( uPtetO \)

The \( uPtetO \) promoters were built using a series of long forward primers in a step-wise amplification of \( gfp\text{mut2} \) that was very similar to the three step PCR methodology used to make \( ptetR(5-8) \) (section 3.2.2.3 and 3.2.2.4) (Figure 3-14 A). All PCR reactions using \( Pwo \) DNA polymerase required 5% DMSO to successfully amplify the \( gfp\text{mut2} \) constructs to make the tet-responsive promoter GFP fusion constructs, \( uPtetO-GFP \). However, sequencing of the completed \( uPtetO-GFP \) constructs revealed that all the sequenced clones contained one or more single base-pair deletions in the \( tetO \) sequences. Therefore \( uPtetO-GFP \) constructs were remade, using another high fidelity DNA polymerase, Accuprime™ \( Pfx \), which did not require DMSO to amplify the \( gfp\text{mut2} \) constructs. Briefly, pONDG was used as template DNA in Step1 to amplify \( gfp\text{mut2} \), using primers \( \text{tetOGFP1} \) and \( \text{tetOGFP5} \) (Figure 3-15 A). In Step 2, forward primers \( \text{tetOGFP2} \), \( \text{tetOGFP3} \) and \( \text{tetOGFP4} \) were used with reverse primer \( \text{tetOGFP5} \) for the constructs \( uPtetO1-GFP \), \( uPtetO2-GFP \) and \( uPtetO3-GFP \) respectively (Figure 3-15 B), and primers \( \text{tetOGFP5} \) and \( \text{tetOGFP6} \) were used in a final PCR, Step 3, to complete the three constructs (Figure 3-15 C). The final 900 bp constructs consisted of a modified core mutant \( ureA \) promoter, containing one or more \( tetO \) binding sites, fused to \( gfp\text{mut2} \), separated by \( NdeI \) cut site, and flanked on both ends by several unique restriction sites (Figure 3-14 B). Each construct was digested with \( BamHI \) and ligated into the vector backbone of similarly digested pBlu_BI plasmid (Benghezal et al., 2010) to generate plasmids pBlu_uPtetO(1-3)-GFP (Table 3-1, pAD314-pAD316) (Figure 3-16).

Correct incorporation of \( uPtetO-GFP \) into pBlu_uPtetO(1-3)-GFP was confirmed by restriction enzyme digest. \( NcoI \) digestion, which cut once in \( gfp\text{mut2} \) to linearize the plasmid, confirmed that only one copy of \( uPtetO-GFP \) had been cloned, and treatment with both \( NcoI \) and \( SacII \) released either a 900 bp or 600 bp fragment depending on the orientation of the \( uPtetO-GFP \) insert (Figure 3-15 D, specific example for pBlu-uPtetO1-GFP). Double digest of clones c1 and c2 showed that the \( uPtetO(1)-GFP \) construct had been cloned in an opposite orientation to clones c3 and c4. Sequencing of several transformants per construct identified at least two clones for each construct that had correct insert sequences.
Figure 3-14 Construction of tet-responsive promoter-GFP fusion constructs, \( uPtetO-GFP \)
Figure 3-15  PCR construction of uPtetO-GFP

A. Construction of uPtetO: Step 1
   Lane 1: Amplification of gfpmut2, annealing temperature 55 °C

B. Construction of uPtetO: Step 2
   Specific conditions
   Lane 1: For uPtetO1, primer tetOGFP2, annealing temperature 50 °C
   Lane 2: For uPtetO2, primer tetOGFP3, annealing temperature 55 °C
   Lane 3: For uPtetO2, primer tetOGFP3, annealing temperature 60 °C
   Lane 4: For uPtetO3, primer tetOGFP4, annealing temperature 55 °C

C. Construction of uPtetO: Step 3, annealing temperature 50 °C
   Lane 1: For uPtetO1
   Lane 2: For uPtetO2
   Lane 3: For uPtetO3

D. Analysis of plasmid isolated from E. coli transformants for pBlu_uPtetO-GFP
   Specific examples are of pBlu_uPtetO1-GFP (pAD314)
   Lane 1 – 3: pBlu_uPtetO1-GFP clone 1
      Lane 1: Uncut
      Lane 2: NcoI
      Lane 3: NcoI and SacII double digest (600 bp and 3.8 kb or 900 bp and 3.5 kb)
   Lane 4 – 6: pBlu_uPtetO1-GFP clone 2
   Lane 7 – 9: pBlu_uPtetO1-GFP clone 3
   Lane 10 – 12: pBlu_uPtetO1-GFP clone 4
Figure 3-16  Construction of pBlu_uPtetO-GFP (pAD314-pAD316)
3.2.4 Construction of GFP reporter strains to characterize the tetracycline regulation of \textit{uPtetO} in \textit{H. pylori}

To test if the \textit{uPtetO} promoters were functional in \textit{H. pylori}, the \textit{uPtetO}(1-3)-GFP constructs were inserted into the \textit{H. pylori} chromosome. Constructs, \textit{uPtetO}(1-3)-GFP, were excised from \textit{pBlu_uPtetO}(1-3)-GFP by double restriction digest with \textit{SalI} and \textit{XbaI} and cloned into similarly digested \textit{pGltDH}, \textit{pTrpA} and \textit{pHdapB} vectors to make plasmids \textit{pGltDH-uPtetO}(1-3)-GFP, \textit{pTrpA-uPtetO}(1-3)-GFP and \textit{pHdapB-uPtetO}(1-3)-GFP respectively (Table 3-1, pAD317-pAD325) (Figure 3-17 and Figure 3-18). Correct incorporation of \textit{uPtetO-GFP} constructs was confirmed by restriction digest. The \textit{pGltDH-uPtetO}(1-3)-GFP and \textit{pTrpA-uPtetO}(1-3)-GFP plasmids were digested with \textit{NcoI}, which cut once in the \textit{gfp}mut2 sequence to linearize the plasmids, and with both \textit{NcoI} and \textit{SacII} to release either a 1.6 kb insert for \textit{pGltDH-uPtetO}(1-3)-GFP (Figure 3-19 A) or a 1.5 kb insert for \textit{pTrpA-uPtetO}(1-3)-GFP (Figure 3-19 B). The \textit{pHdapB-uPtetO}(1-3)-GFP plasmids were digested with \textit{XbaI}, which cut once in the \textit{uPtetO-GFP} sequence to linearize the plasmids, and with both \textit{XbaI} and \textit{HindIII} to excise a 600 bp band corresponding to the upstream flanking arm of the \textit{dapB} locus (Figure 3-19 C). This series of plasmid constructs was used to introduce \textit{uPtetO-GFP} into \textit{H. pylori} X47 by natural transformation of the appropriate X47 recipient strains.

Wild-type X47 and X47 \textit{mdaB::ptetR}(1-6) strains were transformed with \textit{pTrpA-RCAT}, \textit{pGltDH-RCAT} and \textit{pHdapB-RCAT} to generate the respective recipient strains (Table 3-2, hAD8-hAD10 and hAD17-hAD37) (transformation of wild-type X47 with \textit{pTrpA-RCAT} and \textit{pGltDH-RCAT} was done by Carola Schwan) Insertion of \textit{rpsL-cat} into the correct locus was verified by colony PCR. Recipient strains were then transformed with the appropriate \textit{uPtetO}(1-3)-GFP containing plasmids (pAD317-pAD325) to generate control X47 \textit{uPtetO-GFP} strains (Table 3-2, hAD11-hAD16) that expressed GFP but lacked TetRs, and a panel of X47 \textit{ptetR; uPtetO-GFP} strains (Table 3-4, hAD38-hAD73) that expressed both the \textit{tet} repressor and GFP. Transformants were isolated on streptomycin plates and screened for replacement of the \textit{rpsL-cat} selection cassette with \textit{uPtetO-GFP} by colony PCR (as described in 3.2.1.6) (Figure 3-20 A-C). PCR products amplified from the genomic DNA of \textit{uPtetO-GFP} positive clones were 600 bp smaller than the products amplified from the genomic DNA of the recipient strains harbouring \textit{rpsL-cat}.
Figure 3-17  Construction of plasmid pGltDH-uPtetO-GFP (pAD320-pAD322)
Figure 3-18  Plasmids maps of pTrpA-uPtetO-GFP (pAD317-pAD319) and pHdapB-uPtetO-GFP (pAD323-pAD325)
Figure 3-19  Restriction analysis of plasmids products resulting from cloning $uPtetO$-GFP into cloning vectors
Figure 3-19  Restriction analysis of plasmids products resulting from cloning 

\( uPtetO-GFP \) into cloning vectors (adjacent page)

A. Restriction analysis of plasmid isolated from \( E. coli \) transformants for pGltDH-uPtetO-GFP. Specific examples are of pGltHD-uPtetO1-GFP

Lane 1 – 3: pGltHD-uPtetO1-GFP clone 1
  Lane 1: Uncut
  Lane 2: \( NeoI \)
  Lane 3: \( NeoI \) and \( SacII \) double digest (1.6 kb and 4.3 kb)

Lane 4 – 6: pGltHD-uPtetO1-GFP clone 2
Lane 7 – 9: pGltHD-uPtetO1-GFP clone 3
Lane 10 – 12: pGltHD-uPtetO1-GFP clone 4

B. Restriction analysis of plasmid isolated from \( E. coli \) transformants for pTrpA-uPtetO-GFP. Specific examples are of pTrpA-uPtetO1-GFP

Lane 1 – 3: pTrpA-uPtetO1-GFP clone 1
  Lane 1: Uncut
  Lane 2: \( NeoI \)
  Lane 3: \( NeoI \) and \( SacII \) double digest (1.5 kb and 4.1 kb)

Lane 4 – 6: pTrpA-uPtetO1-GFP clone 2
Lane 7 – 9: pTrpA-uPtetO1-GFP clone 3
Lane 10 – 12: pTrpA-uPtetO1-GFP clone 4

C. Restriction analysis of plasmid isolated from \( E. coli \) transformants for pHdapB-uPtetO-GFP. Specific examples are of pHdapB-uPtetO1-GFP

Lane 1 – 3: pHdapB-uPtetO1-GFP clone 1
  Lane 1: Uncut
  Lane 2: \( XbaI \)
  Lane 3: \( XbaI \) and \( HindIII \) double digest (600 bp and 4.9 kb)

Lane 4 – 6: pHdapB-uPtetO1-GFP clone 2
Lane 7 – 9: pHdapB-uPtetO1-GFP clone 3
Lane 10 – 12: pHdapB-uPtetO1-GFP clone 4
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Figure 3-20  PCR analysis of *H. pylori* X47 transformants harbouring *uPtetO-GFP*
Figure 3-20 PCR analysis of *H. pylori* X47 transformants harbouring *uPtetO-GFP* (adjacent page)

A. PCR analysis of *gltDH* locus using primers GltDH1 and GltDH4 on genomic DNA isolated from

Lane 1 – 4: *H. pylori* X47 *gltDH::uPtetO1-GFP* strains  
Lane 5 – 8: *H. pylori* X47 *gltDH::uPtetO2-GFP* strains  
Lane 9: *H. pylori* X47 *gltDH::rpsL-cat* recipient  
Lane 12: *H. pylori* X47 wild-type  

Lanes 1 through 8 are positive for the *uPtetO-GFP* construct. Similar analysis was done to screen the other *H. pylori* X47 strains transformed to insert *uPtetO-GFP* into the *gltDH* locus.

B. PCR analysis of *trpA* locus using primers TrpA1 and TrpA4 on genomic DNA isolated from

Lane 1 – 4: *H. pylori* X47 *trpA::uPtetO1-GFP* strains  
Lane 5 – 8: *H. pylori* X47 *trpA::uPtetO2-GFP* strains  
Lane 9: *H. pylori* X47 *trpA::rpsL-cat* recipient  
Lane 12: *H. pylori* X47 wild-type  

Lanes 1 through 8 are positive for the *uPtetO-GFP* construct. Similar analysis was done to screen the other *H. pylori* X47 strains transformed to insert *uPtetO-GFP* into the *trpA* locus.

C. PCR analysis of *dapB* locus using primers DapB1 and DapB4 on genomic DNA isolated from

Lane 1 – 3: *H. pylori* X47 *dapB::uPtetO1-GFP* strains  
Lane 4 – 6: *H. pylori* X47 *dapB::uPtetO2-GFP* strains  
Lane 7: *H. pylori* X47 *dapB::rpsL-cat* recipient  
Lane 8: *H. pylori* X47 wild-type  

Lanes 1 through 6 are positive for the *uPtetO-GFP* construct. Similar analysis was done to screen the other *H. pylori* X47 strains transformed to insert *uPtetO-GFP* into the *dapB* locus.
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<th>Promoter</th>
<th>Expression state</th>
<th>Tetracycline</th>
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<td>amiE</td>
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<td>OFF</td>
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<tr>
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<td>amiE</td>
<td>ON</td>
<td>OFF</td>
</tr>
<tr>
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<td>X47 mdaB::ptetR1; dapB::uPtetO3-GFP</td>
<td>amiE</td>
<td>ON</td>
<td>OFF</td>
</tr>
<tr>
<td>hAD45</td>
<td>X47 mdaB::ptetR2; gltDH::uPtetO1-GFP</td>
<td>amiE</td>
<td>OFF</td>
<td>ON</td>
</tr>
<tr>
<td>hAD46</td>
<td>X47 mdaB::ptetR2; gltDH::uPtetO2-GFP</td>
<td>amiE</td>
<td>OFF</td>
<td>ON</td>
</tr>
<tr>
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<td>X47 mdaB::ptetR2; trpA::uPtetO1-GFP</td>
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3.3 Tet-regulation of CGT in *H. pylori*

3.3.1 Construction of tetracycline responsive constructs *uPtetO-CGT* and *uPtetO-CGT-HA*

To generate *H. pylori* strains that expressed CGT in a tetracycline responsive manner, the expression of *cgt* was placed under the control of *tet*-promoters, *uPtetO*, (Described in 3.2.3 and Chapter 4). Two variants of CGT were placed under *uPtetO* expression. The first variant was the wild-type *cgt* gene and the second variant was *cgt-HA*, where 33 bp of DNA was added after the *cgt* coding sequence. This variant, CGT-HA, was expressed as the full length CGT protein with a C-terminal HA peptide tag (SFERFEIFPKE). The HA peptide is derived from the influenza hemagglutinin (HA) molecule H1 (aa 110-119) from PR8 strain and is recognized by an antibody produced by the CM1-1 hybridoma cell line (Chianese-Bullock et al., 1998).

3.3.1.1 Construction of plasmids containing *uPtetO-CGT*

DNA encoding *cgt*, HP0421 ORF, was amplified from 26695 genomic DNA using primers tetOCGT1 and tetOCGT2, *Pwo* DNA polymerase and an annealing temperature of 55 °C (Figure 3-21 A). These primers were used to introduce 5′ *Nde*I and 3′ *Sal*I restriction endonuclease cut sites. Plasmids pBlu-uPtetO(1-3)-GFP (Table 3-1, pAD314-pAD316) were digested with *Nde*I and *Sal*I to excise *gfp*mut2 and the plasmid backbones were isolated by agarose gel purification (section 2.2.2.2). The amplified *cgt* PCR product was similarly treated and cloned into the purified plasmid backbones to generate pBlu-uPtetO(1-3)-CGT (Figure 3-22) (Table 3-1, pAD401-pAD403). Correct replacement of *gfp*mut2 with *cgt* was confirmed by restriction enzyme digest with *Nde*I, which cut once to linearize the plasmid, to ensure that only one copy of *cgt* was present, and with both *Nde*I and *Sac*II to release a 1.5 kb insert (Figure 3-21 B). Following the strategy described in section 3.2.3, *uPtetO-CGT* constructs were excised from pBlu_uPtetO(1-3)-CGT by double restriction digest with *Sal*I and *Xba*I and cloned into similarly digested pGltDH, pTrpA and pHdapB vectors to yield a series of plasmid constructs, pGltDH-uPtetO(1-3)-CGT, pTrpA-uPtetO(1-3)-CGT and pHdapB-uPtetO(1-2)-CGT (Figure 3-23 and 3-24) (Table 3-1, pAD404-pAD411 ) which were used to generate *H. pylori* strains that expressed CGT under the control of tetracycline responsive promoters, *uPtetO*(1-3). These plasmids were confirmed by restriction
digest. The pGltDH-uPtetO-CGT and pTrpA-uPtetO-CGT plasmids were digested with \textit{NdeI}, which cut once in the \textit{uPtetO-CGT} sequence to linearize the plasmids, and with both \textit{NdeI} and \textit{SacII} to release a 2.2 kb insert for both pGltDH-uPtetO-CGT (Figure 3-25 A) and pTrpA-uPtetO-CGT (Figure 3-25 B). The pHdapB-uPtetO-CGT plasmids were digested with \textit{NdeI}, which cut once in the \textit{uPtetO-CGT} sequence and once in the plasmid backbone to release a 1.3 kb insert (Figure 3-25 C).

**Figure 3-21** Construction of \textit{uPtetO-CGT}

A. Amplification of \textit{cgt} (HP0421), annealing temperature 55 °C

B. Restriction analysis of plasmid isolated from \textit{E. coli} transformants for pBlu_uPtetO-CGT. Specific examples are of pBlu_uPtetO1-CGT

Lane 1 – 3: vector control – no insert in ligation reaction
- Lane 1: Uncut
- Lane 2: \textit{NdeI}
- Lane 3: \textit{NdeI} and \textit{SacII} double digest

Lane 4 – 6: pBlu_uPtetO1-CGT clone 1
- Lane 4: Uncut
- Lane 5: \textit{NdeI}
- Lane 6: \textit{NdeI} and \textit{SacII} double digest (1.5 kb and 3.4 kb)

Lane 7 – 9: pBlu_uPtetO1-CGT clone 2
- Lane 10 – 12: pBlu_uPtetO1-CGT clone 3
Figure 3-22  Construction of pBlu-uPtetO(1-3)-CGT (pAD401-pAD403)
Figure 3-23  Construction of plasmid pTrpA(1-3)-uP tetO-CGT  
(pAD404-pAD406)
Figure 3-24  Plasmid maps of (A) pGltDH-uPtetO(1-3)-CGT (pAD407-pAD409) and (B) pHdapB-uPtetO(1-2)-CGT (pAD410-pAD411)
Figure 3-25  Construction of transformation plasmids harbouring *uPtetO-CGT*

A. Restriction analysis of plasmid isolated from *E. coli* transformants for pTrpA-uPtetO-CGT. Specific examples are of pTrpA-uPtetO1-CGT
Lane 1 – 3: pTrpA-uPtetO1-CGT clone 1
   Lane 1: Uncut
   Lane 2: *Nde*I
   Lane 3: *Nde*I and *Sac*II double digest (2.2 kb and 3.9 kb)
Lane 4 – 6: pBlu_uPtetO1-CGT clone 2
Lane 7 – 9: pBlu_uPtetO1-CGT clone 3

B. Restriction analysis of plasmid isolated from *E. coli* transformants for pGltDH-uPtetO-CGT. Specific examples are of pGltDH-uPtetO1-CGT
Lane 1 – 3: pGltDH-uPtetO1-CGT clone 1
   Lane 1: Uncut
   Lane 2: *Nde*I
   Lane 3: *Nde*I and *Sac*II double digest (2.2 kb and 4.1 kb)
Lane 4 – 6: pBlu_uPtetO1-CGT clone 2
Lane 7 – 9: pBlu_uPtetO1-CGT clone 3

C. Restriction analysis of plasmid isolated from *E. coli* transformants for pHdapB-uPtetO-CGT. Specific examples are of pHdapB-uPtetO1-CGT
Lane 1 – 2: pHdapB-uPtetO1-CGT clone 1
   Lane 1: Uncut
   Lane 2: *Nde*I digest (1.3 kb and 4.7 kb)
Lane 3 – 4: pHdapB-uPtetO1-CGT clone 2
Lane 5 – 6: pHdapB-uPtetO1-CGT clone 3
3.3.1.2 Construction of plasmids containing $uPtetO$-$CGT$-$HA$

Plasmids harbouring the $uPtetO$-$CGT$-$HA$ construct were successfully made with similar results to those described above. DNA encoding $cgt$ was amplified from 26695 genomic DNA using primers tetOCGT-HA1 and tetOCGT-HA2, $Pwo$ DNA polymerase and an annealing temperature of 55 °C. The primers introduced a 33 bp sequence encoding the HA peptide after the last HP0421 codon and flanking 5′ $NdeI$ and 3′ $SalI$ restriction endonuclease cut sites. Plasmids pAD317-pAD319, and pAD322 were digested with $NdeI$ and $SalI$ to excise $gfp$mut2 and the plasmid backbones were isolated by agarose gel purification. The amplified $cgt$-$HA$ PCR product was similarly treated and cloned into the purified plasmid backbones to generate pTrpA-$uPtetO$(1-3)-CGT-HA and pGltDH-$uPtetO3$-CGT-HA (Table 3-1 pAD412-pAD415) which were used to generate $H. pylori$ strains that expressed a HA-tagged CGT under the control of a tetracycline responsive promoter (Figure 3-26).

3.3.2 Construction of first generation $cgt$ mutant strains

The first strategy used to generate a $cgt$ knockout mutant in strain X47 involved making a markerless mutation by cleanly removing $HP0421$ from the genome, which would permit multiple uses of the $rpsL$-$cat$ cassette for additional DNA manipulations within the same strain. To achieve this, two constructs were made: The first, $HP0421::rpsL$-$cat$, was made to replace a large portion of $HP0421$ with $rpsL$-$cat$ cassette and the second, $HP0421del$, was made to replace the $rpsL$-$cat$ cassette and the remainder of $HP0421$ to generate a final $H. pylori$ strain lacking $cgt$. 

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Figure 3-26 Construction of plasmid pTrpA-uPtetO(1-3)-CGT-HA (pAD412-pAD414) and plasmid map of pGltDH-uPtetO1-CGT-HA (pAD415)
3.3.2.1 **Construction of X 47 HP0421::rpsL-cat-1**

The *HP0421::rpsL-cat* construct was generated by SOE PCR (Figure 3-27). Two 1 kb regions of DNA flanking the first half of the *cgt* (*HP0421*) ORF, arm I and arm II, were amplified from 26695 genomic DNA using primers CGTrcat1 and CGTrcat2, and CGTrcat3 and CGTrcat4 respectively(Figure 3-28 A). The *rpsL-cat* counterselection cassette (middle arm III) was amplified from pENT-RC using primers CGTrcat5 and CGTrcat6 and *Taq* DNA polymerase. Attempts to join all three arms together in one SOE PCR reaction were unsuccessful, yielding a very weak product band corresponding to the size of the PCR product of interest. However, using successive rounds of SOE PCR was successful. Arms I and III and arms II and III were joined using primer pairs CGTrcat1 and CGTrcat6, and CGTrcat4 and CGTrcat5 respectively and an extension time of 100 s for the first 10 cycles, followed by an extension time of 160 s, to yield 2.2 kb and 2.3 kb PCR products (Figure 3-28 B). The PCR products from the first round of SOE PCR were purified and used in the second round of SOE PCR, using extension times of 160 s and 220 s and nested primers CGTrcat7 and CGTrcat8, to generate and amplify a final 3.2 kb (outer primers) or 3.0 kb (nested primers) *HP0421::rpsL-cat* construct (Figure 3-28 C).

Natural transformation of the *H. pylori* strain X47 with *HP0421::rpsL-cat* was performed to obtain the recipient strain X47 *HP0421::rpsL-cat-1*. Correct allelic replacement of the resulting Cm\(^r\) transformants was confirmed by colony PCR (2.2.2.3) using primers CGTrcat1 and CGTrcat4 with an annealing temperature of 55 °C and an extension time of 240 s. PCR analysis of genomic DNA isolated from pure clones amplified PCR products which were 700 bp larger than the PCR products amplified from genomic DNA isolated from the wild-type strain, corresponding to the replacement of half of *HP0421* with the *rpsL-cat* cassette (Figure 3-28 D).
Figure 3-27  PCR construction HP0421 knockout construct, HP0421::rpsL-cat
**Figure 3-28**  Generation of *cgt* mutant X47 HP0421::*rpsL-cat-1

A. PCR amplification of arms I, II and III for HP0421::*rpsL-cat*
   Lane 1 – 2: amplification of arm I
   Specific conditions
   - Lane 1: *Pwo*, Annealing temperature 50 °C, 5% DMSO
   - Lane 2: *Pwo*, Annealing temperature 55 °C, 5% DMSO
   Lane 3 – 4: amplification of arm II
   Specific conditions
   - Lane 3: *Pwo*, Annealing temperature 50 °C, 5% DMSO
   - Lane 4: *Pwo*, Annealing temperature 55 °C, 5% DMSO
   Lane 5 – 6: amplification of arm III
   Specific conditions
   - Lane 5: *Taq*, Annealing temperature 50 °C
   - Lane 6: *Taq*, Annealing temperature 55 °C

B. SOE PCR products joining arms I, II and III for HP0421::*rpsL-cat*
   Lane 1 – 2: Three way SOE PCR reaction
   Specific conditions
   - Lane 1: arm I + II + III, outer primers CGTrcat1 and CGTrcat4
   - Lane 2: arm I + II + III, nested primers CGTrcat7 and CGTrcat8
   Lane 3 – 4: Two way SOE PCR reaction
   Specific conditions
   - Lane 3: arm I + III
   - Lane 4: arm II + III

C. Final SOE PCR products joining arms I, II and III for HP0421::*rpsL-cat*
   Specific conditions
   - Lane 1: outer primers CGTrcat1 and CGTrcat4
   - Lane 2: nested primers CGTrcat7 and CGTrcat8

D. PCR analysis, using primers CGTrcat1 and CGTrcat4, on genomic DNA isolated from
   Lane 1-6: *H. pylori* X47 HP0421::*rpsL-cat-1 mutants, Lane 7: *H. pylori* X47 wild-type.
3.3.2.2 Construction of strain X47 ΔHP0421

In order to make a strain that had the HP0421 ORF completely removed from the genome, a construct composed of DNA regions flanking HP0421 was made. The HP0421del construct was generated by SOE PCR (Figure 3-29). Two 900 bp regions of DNA flanking the cgt ORF (HP0421), arm I and arm II, were amplified from 26695 genomic DNA using primers CGTrcat1 and CGTdel1, and CGTdel2 and CGTdel3 respectively. Arm I could not be amplified using Pwo DNA polymerase under the conditions tested. However, when Pwo was substituted for Taq DNA polymerase, arm I was successfully amplified, with an annealing temperature of 45 °C giving the best yield (Figure 3-30 A). Arm II was successfully amplified as a 900 bp PCR product with Pwo DNA polymerase when 5% DMSO was added to the reaction mixture (Figure 3-30 B). The PCR fragments were joined together by SOE PCR using extension times of 60 s and 120 s, to generate and amplify a final a 1.7 kb (outer primers) or slightly smaller 1.4 kb (nested primers) PCR product, HP0421del (Figure 3-30 C).

Natural transformation of the recipient strain, X47 HP0421::rpsL-cat-1 with the nested HP0421del SOE PCR product generated X47 ΔHP0421, a strain that has HP0421 completely removed from the genome. The resulting Str transformants were screened by colony PCR for loss of HP0421, corresponding to PCR product that was 1.9 kb smaller than the PCR product for the parent X47 HP0421::rpsL-cat-1 strain and 1.2 kb smaller than the PCR product amplified form wild-type X47 genomic DNA (Figure 3-30 D).

3.3.3 Construction of second generation cgt mutant strains

Constructs for making second generation cgt mutant strains were designed to promote the expression of HP0422, the ORF downstream of cgt, under the strong cat promoter in the rpsL-cat cassette by introducing or preserving an internal ribosomal binding site. These constructs were made using a different strategy from the one used to make the constructs for the first generation cgt mutants. This strategy used fewer primers and was very similar to the strategy used to make pGltDH-RCAT, pTrpA-RCAT and pHdapB-RCAT in section (3.2.1) (Figure 3-31 and 3-32). The constructs, pCGT-Δ2-RCAT and pCGT-Δ3-RCAT, required for making the second generation cgt mutants were made in a two-step process. The first step involved making precursor plasmids, pCGT-Δ2 and pCGT-Δ3, which contained 1.7 kb of DNA that flanked the HP0421 ORF.
Figure 3-29  PCR construction HP0421 clean deletion construct, HP0421del
Chapter 3: Plasmid and strain construction

Figure 3-30  Generation of cgt mutant, X47 ΔHP0421

A. PCR amplification of arm I, HP0421del
   Specific conditions
   Lane 1: Taq, Annealing temperature 45 °C
   Lane 2: Taq, Annealing temperature 58.5 °C
   Lane 3: Taq, Annealing temperature 53 °C
   Lane 4: Taq, Annealing temperature 56.5 °C
   Lane 5: Taq, Annealing temperature 60 °C

B. PCR amplification of arm II, HP0421del
   Specific conditions
   Lane 1: Pwo, Annealing temperature 50 °C, 5% DMSO
   Lane 2: Pwo, Annealing temperature 50 °C
   Lane 3: Pwo, Annealing temperature 55 °C, 5% DMSO
   Lane 4: Pwo, Annealing temperature 55 °C

C. SOE PCR products joining arms I and II for HP0421del
   Lane 1 – 2: Two way SOE PCR reaction
   Specific conditions
   Lane 1: outer primers CGTcat1 and CGTdel3 (1.7 kb)
   Lane 2: nested primers CGTcat7 and CGTdel4 (1.4 kb)

D. PCR analysis, using primers CGTcat1 and CGTdel3, on genomic DNA isolated from
   Lane 1-6: H. pylori X47 ΔHP0421 mutants
   Lane 7: H. pylori X47 wild-type
   Lane 8: H. pylori X47 HP0421::rpsL-cat-1 mutant

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Figure 3-31  Construction of plasmid pCGTΔ3 (pAD417)
Figure 3-32 Construction of plasmid pCGTΔ3-RCAT (pAD419)
3.3.3.1 Construction of precursor plasmids, pCGT-Δ2 and pCGT-Δ3

*pCGT-Δ2*: 900 bp region upstream of and including the first 18 bp of the HP0421 ORF and a 1 kb region downstream of and including the last 129 bp of the HP0421 ORF was amplified using primers CGTrcat1 and CGTrcat11, and CGTrcat12 and CGTdel3 respectively (Figure 3-33 A). The two PCR fragments were joined together by SOE PCR, using an extension time of 60 s for the first 10 cycles, followed by the addition of primers CGTrcat1 and CGTdel3 and an increase of extension time to 120 s (Figure 3-33 B). The SOE PCR for *CGT-Δ2* gave the product of interest in addition to several smaller bands, and therefore had to be purified away from the contaminating bands by gel extraction. The resulting 1.9 kb PCR product contained a unique *Bam*HI site separating the two flanking DNA sequences of HP0421. The PCR product was cloned into pGEM®-T Easy vector (section 2.2.2.4 – DNA ligations) to give pCGT-Δ2, (Figure 3-34 A).

*pCGT-Δ3*: A similar strategy was used as described for pCGT-Δ2. The key differences in this construct were that a 900 bp region upstream of and including the first 3 bp (ATG) of the HP0421 ORF and a 850 bp region downstream of the HP0421, starting from 3 bp before the HP0422 ORF, were amplified using primers CGTrcat1 and CGTrcat13, and CGTrcat14 and CGTdel3 respectively (Figure 3-33 A). The two PCR fragments were joined together by SOE PCR to give a single major PCR product of 1.6 kb, (Figure 3-33 B) which was cloned into pGEM®-T Easy vector to give pCGT-Δ3 (Figure 3-31).

Plasmids isolated from *E. coli* transformants were analyzed by restriction enzyme digest for correct incorporation of *CGT-Δ2* and *CGT-Δ3*. Single digest with *Bam*HI confirmed that the unique *Bam*HI site inserted between the two flanking regions was intact, and the double restriction digests gave the expected restriction patterns, confirming that all the screened clones had the correct plasmid size of 4.9 kb (Figure 3-33 C - specific example for pCGT-Δ2).
Figure 3-33  Construction of pCGT-Δ2 and pCGT-Δ3

A. PCR amplification of arm I and II, for CGT-Δ2 and CGT-Δ3
   Lane 1 – 2: Amplification of arm I, CGT-Δ2
   Specific conditions
      Lane 1: Pwo, Annealing temperature 55 °C, 5% DMSO
      Lane 2: Pwo, Annealing temperature 55 °C
   Lane 3 – 4: Amplification of arm II, CGT-Δ2
   Lane 5 – 6: Amplification of arm I, CGT-Δ3
   Lane 7 – 8: Amplification of arm II, CGT Δ3

B. SOE PCR products from joining arms I and II
   Lane 1: arm I + arm II for CGT-Δ2
   Lane 2: arm I + arm II for CGT-Δ3

C. Restriction analysis of plasmid isolated from E. coli transformants for pCGT-Δ2
   Lane 1 – 3: pCGT-Δ2clone 1
   Specific conditions
      Lane 1: BamHI digest (4.9 kb)
      Lane 2: BamHI and NcoI double digest (940 bp and 4.0 kb)
      Lane 3: SacI and SacII double digest (2.0 kb and 2.9 kb)
   Lane 4 – 6: pCGT-Δ2 clone 2
   Lane 7 – 9: pCGT-Δ2 clone 3
Construction of CGT knockout plasmids, pCGT-Δ2-RCAT and pCGT-Δ3-RCAT

The second step in making the CGT knockout plasmids, pCGT-Δ2-RCAT and pCGT-Δ3-RCAT, involved cloning the rpsL-cat counterselection cassette into the unique BamHI site between the HP0421 flanking sequences. The rpsL-cat counterselection cassette was excised from pENT-RC, using BamHI restriction endonuclease, gel purified and ligated into the unique BamHI site of pCGT-Δ2 and pCGT-Δ3 to generate pCGT-Δ2-RCAT and pCGT-Δ3-RCAT respectively (Figure 3-32 and Figure 3-34 B). This cloning strategy required rpsL-cat to be inserted in the forward orientation; however rpsL-cat could be cloned into pCGT-Δ2 and pCGT-Δ3 in two possible orientations and therefore E. coli transformants were screened for correct plasmid size and for correct orientation of the counter selection cassette. Restriction endonuclease digest of purified plasmids identified that clones 1 and 2 for pCGT-Δ2-RCAT were the correct size, containing only one insert, while clone 3 was too big, containing an additional copy of rpsL-cat (Figure 3-35 A - specific example for pCGT-Δ2).
Figure 3-35  Construction of pCGT-Δ2-RCAT and pCGT-Δ3-RCAT

A. Restriction analysis of plasmid isolated from *E. coli* transformants for pCGT-Δ2-RCAT
   Lane 1 – 2: pCGT-Δ2-RCAT clone 1
   Specific conditions
      Lane 1: *Cla*I and *Sac*II double digest (2.2 kb and 4.1 kb)
      Lane 2: *Cla*I and *Sal*I double digest (1.1 kb and 5.2 kb)
   Lane 3 – 4: pCGT-Δ2-RCAT clone 2
   Lane 5 – 6: pCGT-Δ2-RCAT clone 3

B. Restriction analysis of PCR products amplified from pCGT-Δ2-RCAT and pCGT-Δ3-RCAT clones
   Lane 1 – 2: pCGT-Δ3-RCAT clone 1
   Specific conditions
      Lane 1: Undigested
      Lane 2: *Cla*I digest (expect 400 bp and 2.0 kb)
   Lane 3 – 4: pCGT-Δ3-RCAT clone 2
   Lane 5 – 6: pCGT-Δ3-RCAT clone 3
   Lane 7 – 8: pCGT-Δ3-RCAT clone 4
   Lane 9 – 10: pCGT-Δ2-RCAT clone 1
   Specific conditions
      Lane 1: Undigested
      Lane 2: *Cla*I digest (expect 550 bp and 2.0 kb)
   Lane 11 – 12: pCGT-Δ2-RCAT clone 2
   Lane 13 – 14: pCGT-Δ2-RCAT clone 3
   Lane 15 – 16: pCGT-Δ2-RCAT clone 4
To determine the orientation of the *rpsL-cat* cassette relative to the portion of the HP0422 ORF in the pCGT-Δ2-RCAT and pCGT-Δ3-RCAT constructs, purified plasmids were used as templates in a standard PCR reaction, using *Taq* DNA polymerase, primers CGTrcat7 and CGTrcat8 with an annealing temperature of 55 °C and a 240 s extension time, to amplify *rpsL-cat* along with 750 bp of arm I and only 440 bp or 310 bp of arm II. The resulting PCR product was purified and treated with *Cla*I restriction endonuclease for 1 h at 37 °C and reaction products were analysed by agarose gel electrophoresis. For plasmids harbouring the counterselection cassette in the correct forward orientation, *Cla*I digestion of the PCR product would cut once within the amplified DNA sequence, 110 bp from the 3′ end of the *rpsL-cat* cassette, and generate a smaller DNA fragment that would be 550 bp in size for the pCGT-Δ2-RCAT construct and 400 bp in size for the pCGT-Δ3-RCAT construct. However, if the *rpsL-cat* cassette was in the reverse orientation, then the smaller DNA fragment generated by *Cla*I digestion would be much larger, approximately 850 bp in size for both constructs. Analysis of the DNA fragments produced by PCR amplification and subsequent treatment with *Cla*I identified that clones 1 and 2 for pCGT-Δ2-RCAT and clones 2, 3 and 4 for pCGT-Δ3-RCAT, were the only clones containing a single copy of *rpsL-cat* and observation of the smaller DNA fragments, 550bp and 400 bp in size, in the lanes loaded with samples treated with *Cla*I, demonstrated that these 5 clones contained *rpsL-cat* in the desired forward orientation (Figure 3-35 B).

### 3.3.3.3 Generation of second generation cgt mutants, X47 HP0421::rpsL-cat-2 and X47 HP0421::rpsL-cat-3

Natural transformation of the wild-type X47 strain, with non-replicating plasmids pCGT-Δ2-RCAT and pCGT-Δ3-RCAT generated strain X47 HP0421::rpsL-cat-2 and HP0421::rpsL-cat-3 respectively. The resulting Cm⁷ transformants were screened by colony PCR for replacement of HP0421 with *rpsL-cat*, corresponding to PCR product that was 400 bp and 300 bp larger than the PCR 2.9 kb product amplified form wild-type X47 genomic DNA (Figure 3-36). The difference in PCR product size was difficult to clearly resolve under standard DNA gel electrophoresis conditions and therefore gels were run at 70 V for 2 h to improve the resolution of these PCR products.
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Figure 3-36  Colony PCR of second generation cgt mutants

PCR analysis, using primers CGTcat1 and CGTdel3, on genomic DNA isolated from:
Lane 1, 2, 6 and 7: *H. pylori* X47 HP0421::rpsL-cat-2 mutants
Lane 3, 4, 8 - 11: *H. pylori* X47 HP0421::rpsL-cat-3 mutants
Lane 5 and 12: *H. pylori* X47 wild-type strain

3.3.4 Construction of conditional cgt mutant strains

3.3.4.1 Complementation of first generation cgt mutants

X47 ΔHP0421 strain was genetically complemented by insertion of *uPtetO-CGT* constructs at either the gltDH, trpA or dapB locus. *H. pylori* strain X47 ΔHP0421 was transformed with either pGltDH-RCAT, pTrpA-RCAT or pHdapB-RCAT (Table 3-1, pAD302, pAD304, pAD306) to generate recipient strains X47 ΔHP0421; gltDH::rpsL-cat, X47 ΔHP0421; trpA::rpsL-cat and X47 ΔHP0421; dapB::rpsL-cat respectively (Table 3-2, hAD76-hAD78). Cm' transformants were screened for correct integration of *rpsL-cat* as described in section 3.2.1.6. Recipient strains were then transformed with the appropriate *uPtetO(1-2)-CGT* containing plasmids (Table 3-1, pAD404-pAD411) to obtain a panel of X47 ΔHP0421; *uPtetO-CGT* strains (Table 3-2, hAD79-hAD84), which contained one copy of cgt expressed under the control of a tet-promoter, *uPtetO(1-2)*. Transformants were isolated on streptomycin plates and screened by colony PCR for correct replacement of *rpsL-cat* with *uPtetO-CGT* as described in section 3.2.1.6 (Figure 3-37).
3.3.4.2 Complementation of second generation cgt mutants

Since the counterselection cassette remained in the second generation cgt mutant strains, additional DNA manipulations using rpsL-cat cassette could not be done in these strains. Complementing these strains by introducing a copy of cgt on a plasmid was not feasible, as X47 does not easily take up plasmids (Ondek Pty. Ltd., unpublished data).

A second counterselectable marker for genetic manipulation of H. pylori was not available and consequently any additional genetic manipulations of second generation cgt mutant strains using rpsL-cat replacement strategy had to be done before the HP0421 mutation was made. Therefore, to generate genetically complemented second generation cgt mutants, a second copy of cgt, in the form of uPtetO-CGT, was first inserted at either the gltDH or trpA locus followed by replacement of the native cgt gene in the HP0421 locus by rpsL-cat using pCGT-Δ2-RCAT and pCGT-Δ3-RCAT. Though this was not the optimal strategy, great care was taken to ensure that the desired mutations were made and that they were stable.

Natural transformation of H. pylori recipient strains, X47 gltDH::rpsL-cat and X47 trpA::rpsL-cat (Table 3-2, hAD8 and hAD9) with the appropriate uPtetO-CGT containing plasmid (Table 3-1, pAD404 –hAD409) was performed to generate H. pylori strains expressing two copies of cgt, X47 gltDH::uPtetO(1-3)-CGT and X47 trpA::uPtetO(1-3)-CGT (Table 3-2, hAD85-hAD90). Transformants were isolated on streptomycin plates and screened by colony PCR for correct replacement of rpsL-cat with uPtetO-CGT as described in section 3.3.4.1. These six strains were then transformed using either pCGT-Δ2-RCAT or pCGT-Δ3-RCAT to generate a panel of complemented second generation CGT mutants, X47 gltDH::uPtetO(1-3)-CGT; HP0421::rpsL-cat-(2/3) and X47 trpA::uPtetO(1-3)-CGT; HP0421::rpsL-cat-(2/3) (Table 3-2, hAD112-hAD121). Resulting Cm<sup>r</sup> transformants were characterized by PCR to ensure that uPtetO-CGT was still at the recipient locus and that rpsL-cat had replaced the native HP0421 ORF.
Figure 3-37  PCR analysis of HP0421 deletion mutant complemented with uPtetO-CGT at foreign locus

A. PCR analysis of gltDH locus using primers GltDH1 and GltDH4 on genomic DNA isolated from
   Lane 1: H. pylori X47 wild-type strain
   Lane 2: H. pylori X47 ΔHP0421; gltDH::rpsL-cat recipient strain
   Lane 3 – 6: H. pylori X47 ΔHP0421; gltDH::uPtetO1-CGT strains
   Lane 7 – 10: H. pylori X47 ΔHP0421; gltDH::uPtetO2-CGT strains
   Lanes 3 through 10 are positive for the uPtetO-CGT construct. Similar analysis was done to screen other H. pylori X47 transformants with uPtetO-CGT inserted into the gltDH locus.

B. PCR analysis of gltDH locus using internal primer tetOCGT1 and flanking primer GltDH4 on genomic DNA isolated from
   Lane 1 – 4: H. pylori X47 ΔHP0421; gltDH::uPtetO1-CGT strains
   Lane 5 – 8: H. pylori X47 ΔHP0421; gltDH::uPtetO2-CGT strains
   Lane 9: H. pylori X47 ΔHP0421; gltDH::rpsL-cat recipient strain
   Lane 10: H. pylori X47 wild-type strain
   Lanes 1 through 8 are positive for the uPtetO-CGT construct. Similar analysis was done to screen other H. pylori X47 transformants with uPtetO-CGT inserted into the gltDH locus.
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C. PCR analysis of trpA locus using primers TrpA1 and TrpA4 on genomic DNA isolated from
   Lane 1: *H. pylori* X47 wild-type strain  
   Lane 2: *H. pylori* X47 ΔHP0421; trpA::rpsL-cat recipient strain  
   Lane 3 – 6: *H. pylori* X47 ΔHP0421; trpA::uPtetO1-CGT strains  
   Lane 7 – 10: *H. pylori* X47 ΔHP0421; trpA::uPtetO2-CGT strains  

Lanes 3 through 10 are positive for the uPtetO-CGT construct. Similar analysis was done to screen other *H. pylori* X47 transformants with uPtetO-CGT inserted into the trpA locus.

D. PCR analysis of trpA locus using internal primer tetOCGT1 and flanking primer TrpA4 on genomic DNA isolated from
   Lane 1 – 4: *H. pylori* X47 ΔHP0421; trpA::uPtetO1-CGT strains  
   Lane 5 – 8: *H. pylori* X47 ΔHP0421; trpA::uPtetO2-CGT strains  
   Lane 9: *H. pylori* X47 ΔHP0421; trpA::rpsL-cat recipient strain  
   Lane 10: *H. pylori* X47 wild-type strain  

Lanes 1 through 8 are positive for the uPtetO-CGT construct. Similar analysis was done to screen other *H. pylori* X47 transformants with uPtetO-CGT inserted into the trpA locus.

E. PCR analysis of dapB locus using primers DapB1 and DapB4 on genomic DNA isolated from
   Lane 1 – 4: *H. pylori* X47 ΔHP0421; dapB::uPtetO1-CGT strains  
   Lane 5 – 8: *H. pylori* X47 ΔHP0421; dapB::uPtetO2-CGT strains  
   Lane 9: *H. pylori* X47 ΔHP0421; dapB::rpsL-cat recipient strain  
   Lane 10: *H. pylori* X47 wild-type strain  

Lanes 1 through 8 are positive for the uPtetO-CGT construct.

F. PCR analysis of dapB locus using internal primer tetOCGT1 and flanking primer DapB4 on genomic DNA isolated from
   Lane 1 – 4: *H. pylori* X47 ΔHP0421; dapB::uPtetO1-CGT strains  
   Lane 5 – 8: *H. pylori* X47 ΔHP0421; dapB::uPtetO2-CGT strains  
   Lane 9: *H. pylori* X47 ΔHP0421; dapB::rpsL-cat recipient strain  
   Lane 10: *H. pylori* X47 wild-type strain  

Lanes 1 through 8 are positive for the uPtetO-CGT construct.
3.3.4.3 Construction of conditional cgt mutants

Similarly, tet-responsive CGT expressing strains were made as described in 3.3.4.2. Briefly, H. pylori recipient strains expressing TetRs, (Table 3-2, hAD17- hAD29) were transformed with the appropriate uPtetO-CGT containing plasmid (Table 3-1, pAD404-pAD409) to generate H. pylori strains expressing TetRs and two copies of cgt, (Table 3-2, hAD91-hAD109). These strains were then transformed using either pCGT-Δ2-RCAT or pCGT-Δ3-RCAT to generate a panel of tetracycline responsive complemented second generation cgt mutants (Table 3-2, hAD122-hAD148).

3.3.4.4 Construction of H. pylori mutants expressing CGT-HA conditionally

The same strategy described in section 3.3.4.3 was used to generate H. pylori strains that expressed CGT tagged with a C-terminal HA tag from either the trpA or gltDH locus. H. pylori recipient strains expressing TetRs (Table 3-2, hAD18-hAD29) were transformed with the appropriate uPtetO-CGT-HA containing plasmid (Table 3-1, pAD412-pAD415) to generate H. pylori strains expressing TetRs and one copy of cgt and one copy of cgt-HA, (Table 3-5, hAD162-hAD169). Control strains (Table 3-5, hAD158-hAD161), that lack TetRs, were made by transforming X47 GltDH::rpsL-cat and X47 trpA::rpsL-cat (Table 3-2, hAD8 and hAD9) with the appropriate uPtetO-CGT-HA containing plasmid. A subset of strains expressing both the native cgt and cgt-HA underwent a final transformation using either pCGT-Δ2-RCAT or pCGT-Δ3-RCAT to generate a panel of X47 strains that only express the HA-tagged form of CGT either constitutively (Table 3-5, hAD170 and hAD171) or in a tetracycline responsive manner (Table 3-5, hAD172-hAD175).

3.3.5 Quantifying wild-type levels of HP0421 expression

A construct, HP0421-HA, composed of the HA coding sequence fused to the 3’ end of the HP0421 ORF and flanked by DNA sequences located upstream and downstream of the HP0421 locus was made for generate a X47 strain that expressed CGT tagged with a C-terminal HA peptide from the cgt native locus. The HP0421-HA construct was generated by SOE PCR (Figure 3-38). Two 900 bp regions of DNA flanking the cgt (HP0421) ORF, arm I and arm II, were amplified from X47 genomic DNA using primers CGTrcat1 and CGT-HA1, and CGT-HA2 and CGTdel3 respectively (Figure 3-39 A). The HA-tagged cgt sequence, cgt-HA (middle arm III), was amplified from pTrpA-uPtetO1-CGT-HA using primers CGT-HA3 and CGT-HA4
(Figure 3-39 B). The three PCR fragments were joined together by successive rounds of SOE PCR. Arms I and III, and arms II and III were joined using primer pairs CGTrcat1 and CGT-HA4, and CGT-HA3 and CGTdel3, and an extension time of 100 s for the first 10 cycles, followed by an extension time of 160 s (Figure 3-39 C). The 2.1 kb PCR products from the first round of SOE PCR were purified and used in the second round of SOE PCR, using extension times of 160 s and 220 s to obtain the final 3.0 kb (outer primers) or 2.7 kb (nested primers) HP0421-HA construct (Figure 3-39 D).

Natural transformation of the recipient strain, X47 HP0421::rpsL-cat-3, with the nested SOE PCR product generated strain X47 HP0421::HA (Table 3-5, hAD157), were rpsL-cat was replaced with the HP0421 ORF followed by 33 bp encoding the HA peptide. Correct allelic replacement of the resulting Str^r, Cm^s transformants was confirmed by colony PCR using primers CGTrcat3 and CGTrcat8 with an annealing temperature of 55 °C and an extension time of 75 s, to amplify PCR products that were similar in size to the PCR products amplified from genomic DNA isolated from the wild-type strain, corresponding to restoration of the HP0421 ORF (Figure 3-39 E).
Figure 3-38 Construction of HP0421-HA
A. PCR amplification of arms I and II for HP0421-HA
   Lane 1 – 5: amplification of arm I using *Pwo* and an annealing temperature gradient of 45 °C to 57 °C
   Lane 6 – 10: amplification of arm II using *Pwo* and an annealing temperature gradient of 45 °C to 57 °C

B. PCR amplification of arms III for HP0421-HA
   Lane 1 – 5: amplification of arm III using *Pwo* and an annealing temperature gradient of 45 °C to 57 °C

C. SOE PCR products joining arms I, II and III for HP0421-HA
   Specific conditions
   Lane 1: arm I + III
   Lane 2: arm II + III

D. Final SOE PCR products joining arms I, II and III for HP0421-HA
   Specific conditions
   Lane 1: outer primers CGTrcat1 and CGTdel3
   Lane 2: nested primers CGTrcat7 and CGTdel4

E. PCR analysis, using internal primer CGTrcat3 and flanking primer CGTrcat8, on genomic DNA isolated from
   Lane 1 – 4: *H. pylori* X47 HP0421::HA
   Lane 5: *H. pylori* cgt mutant X47 HP0421::rpsL-cat-3
   Lane 6: *H. pylori* X47 wild-type strain
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<th>Strain name</th>
<th>Description</th>
<th>Source or reference</th>
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<td>hAD157</td>
<td>X47 HP0421-HA</td>
<td>39 bases encoding for the CM-1 HA peptide inserted before the HP0421 ORF stop codon</td>
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<td>hAD158</td>
<td>X47 trpA::uPtetO1-CGT-HA</td>
<td>uPtetO1-CGT-HA inserted into HP1277</td>
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<td>hAD159</td>
<td>X47 trpA::uPtetO2-CGT-HA</td>
<td>uPtetO2-CGT-HA inserted into HP1277</td>
<td>This work</td>
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<tr>
<td>hAD160</td>
<td>X47 trpA::uPtetO3-CGT-HA</td>
<td>uPtetO3-CGT-HA inserted into HP1277</td>
<td>This work</td>
</tr>
<tr>
<td>hAD161</td>
<td>X47 gltDH::uPtetO3-CGT-HA</td>
<td>uPtetO3-CGT-HA inserted between HP0379 and HP380</td>
<td>This work</td>
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<tr>
<td>hAD162</td>
<td>X47 mdaB::ptetR(2); trpA::uPtetO1-CGT-HA</td>
<td>promoter-tetR2 inserted between HP630 and HP631; uPtetO1-CGT-HA inserted into HP1277</td>
<td>This work</td>
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<td>hAD163</td>
<td>X47 mdaB::ptetR(2); trpA::uPtetO2-CGT-HA</td>
<td>promoter-tetR2 inserted between HP630 and HP631; uPtetO2-CGT-HA inserted into HP1277</td>
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<tr>
<td>hAD164</td>
<td>X47 mdaB::ptetR(2); trpA::uPtetO3-CGT-HA</td>
<td>promoter-tetR2 inserted between HP630 and HP631; uPtetO3-CGT-HA inserted into HP1277</td>
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<td>hAD165</td>
<td>X47 mdaB::ptetR(2); gltDH::uPtetO3-CGT-HA</td>
<td>promoter-tetR2 inserted between HP0379 and HP380</td>
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<td>hAD166</td>
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<td>X47 mdaB::ptetR(4); gltDH::uPtetO3-CGT-HA</td>
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<td>hAD170</td>
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<td>uPtetO3-CGT-HA inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
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<td>hAD171</td>
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<td>uPtetO3-CGT-HA inserted between HP0379 and HP380, complete replacement of HP0421 with rpsL-cat</td>
<td>This work</td>
</tr>
<tr>
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<td>Strain name</td>
<td>Description</td>
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<td>hAD172</td>
<td>X47 mdaB::ptetR(2); HP0421::rpsL-cat-3</td>
<td>trpA::uPtetO3-CGT-HA; promoter-tetR2 inserted between HP630 and HP631; uPtetO3-CGT-HA inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
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<td>X47 mdaB::ptetR(2); HP0421::rpsL-cat-3</td>
<td>glutDH::uPtetO3-CGT-HA; promoter-tetR2 inserted between HP630 and HP631; uPtetO3-CGT-HA inserted between HP0379 and HP380, complete replacement of HP0421 with rpsL-cat</td>
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<td>hAD174</td>
<td>X47 mdaB::ptetR(4); HP0421::rpsL-cat-3</td>
<td>trpA::uPtetO3-CGT-HA; promoter-tetR4 inserted between HP630 and HP631; uPtetO3-CGT-HA inserted into HP1277, complete replacement of HP0421 with rpsL-cat</td>
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<td>hAD175</td>
<td>X47 mdaB::ptetR(4); HP0421::rpsL-cat-3</td>
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3.4 **Tet-regulation of urease in *H. pylori***

3.4.1 **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<sub>ureA</sub>, were replaced with *tetO* sequences to generate several derivatives of P<sub>ureA</sub>, *urePtetO*(I-V). These promoter constructs were used to replace the native urease promoter, through a two-step transformation approach that utilized the *rpsL-cat* counterselection cassette.

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

A construct, *ureA::rpsL-cat*, composed of the counterselection cassette flanked by DNA homologous to region of the *ureA* locus was made by short multiple fusion PCR and used to generate recipient strains in which *ureA* and P<sub>ureA</sub> were replaced with *rpsL-cat*. (Figure 3-40) Two 1 kb regions flanking *ureA* (HP0073) and P<sub>ureA</sub>, arm I and arm II, were amplified from 26695 genomic DNA using *Pwo* DNA polymerase and primers *ureArcat1* and *ureArcat2*, and *ureArcat3* and *ureArcat4* respectively (Figure 3-41 A). The *rpsL-cat* selection cassette (middle arm III) was amplified from pENT-RC using *Taq* DNA polymerase and primers *ureArcat5* and *ureArcat6*. Attempts to join all three arms together in a single SOE PCR reaction were unsuccessful, yielding a very weak product band corresponding to the size of interest and multiple non-specific bands. However, SOE PCR reactions joining adjacent arms, using primer pairs *ureArCat1* and *ureArCat6*, and *ureArCat4* and *ureArCat5*, and an extension time of 100 s for the first 10 cycles, followed by an extension time of 160 s, successfully yielded 2.4 kb PCR products (Figure 3-41 B). The PCR products from the first round of SOE PCR were purified and used in the second round of SOE PCR, using an extension times of 160 s and 220 s and nested primers *ureArCat7* and *ureArCat8*, to generate and amplify a final 3.4 kb PCR product, *ureA::rpsL-cat* (Figure 3-41 C). Natural transformation of the *H. pylori* strain X47 with the *ureA::rpsL-cat* PCR construct was performed to obtain the recipient strain X47 *ureA::rpsL-cat* (Table 3-2, hAD176). Transformants isolated on chloramphenicol plates were urease negative and PCR analysis of purified genomic DNA amplified PCR products which were 1.0 kb larger than the PCR products amplified from the wild-type strain, corresponding to the replacement of *ureA* and its promoter with the *rpsL-cat* cassette.
Figure 3-40  Diagram of PCR construction strategy for \textit{ureA::rpsL-cat}
Figure 3-41  PCR construction of *ureA::rpsL-cat*

A. Amplification of arms I, II and III for *ureA::rpsL-cat*
   Lane 1 – 2: amplification of arm I
   Specific conditions
   Lane 1: *Pwo*, Annealing temperature 55 °C, 5 % DMSO
   Lane 2: *Pwo*, Annealing temperature 55 °C
   Lane 3 – 4: amplification of arm II
   Specific conditions
   Lane 3: *Pwo*, Annealing temperature 55 °C, 5 % DMSO
   Lane 4: *Pwo*, Annealing temperature 55 °C
   Lane 5 – 6: amplification of arm III
   Specific conditions
   Lane 5: *Taq*, Annealing temperature 50 °C
   Lane 6: *Taq*, Annealing temperature 55 °C

B. SOE PCR products joining arms I, II and III for *ureA::rpsL-cat*
   Lane 1 – 2: Three way SOE PCR reaction
   Specific conditions
   Lane 1: arm I + II + III, outer primers ureArcat1 and ureArcat4
   Lane 2: arm I + II + III, nested primers ureAcat7 and ureArcat8
   Lane 3 – 4: Two way SOE PCR reaction
   Specific conditions
   Lane 3: arm I + III
   Lane 4: arm II + III

C. Final SOE PCR products joining arms I, II and III for *ureA::rpsL-cat*
   Lane 1 – 2: Two way SOE PCR reaction
   Specific conditions
   Lane 1: outer primers ureArcat1 and ureArcat4
   Lane 2: nested primers ureAcat7 and ureArcat8
3.4.1.2 Reconstruction of ureA promoter to incorporate tetO sites

Several tetO modified ureA promoter constructs (urePtetO-I-V), containing up to three tetO sites in different locations, were constructed by SOE PCR. The primer pairs used to make each urePtetO construct are listed in (Table 3-6). Briefly, a 1 kb fragment upstream, arm I, and a 1.5 kb fragment downstream, arm II, of P_ureA were amplified separately using Pwo DNA polymerase (Figure 3-42). Only four of the eight required fragments were successfully amplified using Pwo DNA polymerase and 5% DMSO, (Figure 3-43 A). The annealing temperature of the Pwo PCR reactions was varied from 45 °C to 60 °C in an attempt to optimize the reaction conditions and amplify the remaining fragments, however this was unsuccessful. The remaining fragments were successfully obtained when Pwo was substituted with Taq DNA polymerase (Figure 3-43 B). Long primer tails were used to reconstruct the ureA promoter region upon fusion of arms I and II by SOE PCR, with extension times of 120 s and 180 s. Primers ureArcat7 and ureArcat8 were used to amplify the final 2.5 kb products, urePtetO(I-V) (Figure 3-43 C) and sequencing confirmed that the modified ureA promoters were correctly reconstructed.

<table>
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<th>Construct</th>
<th>arm I - upstream</th>
<th>arm II - downstream</th>
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<tbody>
<tr>
<td>urePtetOI</td>
<td>ureArcat1 &amp; ureAtetO1</td>
<td>ureAtetO2 &amp; ureArcat4</td>
</tr>
<tr>
<td>urePtetOII</td>
<td>ureArcat1 &amp; ureAtetO3</td>
<td>ureAtetO2 &amp; ureArcat4</td>
</tr>
<tr>
<td>urePtetOIII</td>
<td>ureArcat1 &amp; ureAtetO4</td>
<td>ureAtetO5 &amp; ureArcat4</td>
</tr>
<tr>
<td>urePtetOIV</td>
<td>ureArcat1 &amp; ureAtetO6</td>
<td>ureAtetO7 &amp; ureArcat4</td>
</tr>
<tr>
<td>urePtetOV</td>
<td>ureArcat1 &amp; ureAtetO8</td>
<td>ureAtetO7 &amp; ureArcat4</td>
</tr>
</tbody>
</table>

Natural transformation of the recipient strain X47 ureA::rpsL-cat with urePtetO(I-V) PCR constructs resulted in replacement of the rpsL-cat with urePtetO and restoration of ureA to generate X47 urePtetO(I-V) (Table 3-2, hAD177-hAD181). Correct allelic replacement of the resulting Str^r transformants was confirmed by colony PCR using primers ureAP1 and ureArcat8 (Figure 3-44) and by sequencing using primer urePseq.
Figure 3-42  Diagram of PCR construction strategy for *urePtetO*

*urePtetO* product for transformation
Figure 3-43  PCR construction of *urePtetO*

A. Amplification of arms I and II for *urePtetO* using *Pwo*, annealing temperature 55 °C and 5% DMSO

Lane 1: arm I, *urePtetO*I
Lane 2: arm II, *urePtetO*I and *urePtetO*II
Lane 3: arm I, *urePtetO*II
Lane 4: arm I, *urePtetO*III
Lane 5: arm II, *urePtetO*III
Lane 6: arm I, *urePtetO*IV
Lane 7: arm II, *urePtetO*IV and *urePtetO*V
Lane 8: arm I, *urePtetO*V

Only four out of eight PCR reactions amplified the product of interest.

B. Amplification of arms I and II for *urePtetO* using *Tag*, annealing temperature 50 °C

Lane 1: arm I, *urePtetO*I
Lane 2: arm I, *urePtetO*III
Lane 3: arm II, *urePtetO*III
Lane 4: arm I, *urePtetO*IV
Lane 5: arm II, *urePtetO*IV and *urePtetO*V

C. SOE PCR products joining arms I and II for *urePtetO* using primers ureArcat7 and ureArcat8

Lane 1: *urePtetO*I
Lane 2: *urePtetO*II
Lane 3: *urePtetO*III
Lane 4: *urePtetO*IV
Lane 5: *urePtetO*V
3.4.2 Construction of conditional urease knockout strains

Conditional urease strains were generated as follows.  *H. pylori* strains expressing TetRs, *X47 mdaB::ptetR*(1-7) (Table 3-2, hAD1-hAD7) were transformed into recipient strains by natural transformation with the *ureA::rpsL-cat* PCR construct to generate *X47 mdaB::ptetR*(1-7); *ureA::rpsL-cat* (Table 3-2, hAD182-hAD188). These recipient strains were in turn transformed with the PCR constructs, *urePtetO*(I-V), to generate a set of conditional urease knockout strains, *X47 mdaB::ptetR*(1-7); *urePtetO*(I-V) strains (Table 3-7, hAD189-hAD221). Strains expressing TetR were designated Tet-ON strains and strains expressing revTetR were designated as Tet-OFF strains. Transformants were screened for appropriate urease phenotype by patching colonies onto urease plates (6.3.1.1) and confirmed by colony PCR.
## Table 3-7  X47 strains with tet-responsive ureA and ureB expression

<table>
<thead>
<tr>
<th>AD#</th>
<th>Strain name</th>
<th>Promoter</th>
<th>Expression state</th>
<th>Tetracycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAD189</td>
<td>X47 mdaB::ptetR1; urePtetOI</td>
<td>amiE</td>
<td>ON</td>
<td>ON*</td>
</tr>
<tr>
<td>hAD190</td>
<td>X47 mdaB::ptetR1; urePtetOII</td>
<td>amiE</td>
<td>ON</td>
<td>ON*</td>
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<tr>
<td>hAD191</td>
<td>X47 mdaB::ptetR1; urePtetOIII</td>
<td>amiE</td>
<td>ON</td>
<td>ON*</td>
</tr>
<tr>
<td>hAD192</td>
<td>X47 mdaB::ptetR1; urePtetOIV</td>
<td>amiE</td>
<td>ON</td>
<td>ON*</td>
</tr>
<tr>
<td>hAD193</td>
<td>X47 mdaB::ptetR1; urePtetOV</td>
<td>amiE</td>
<td>ON</td>
<td>ON*</td>
</tr>
<tr>
<td>hAD194</td>
<td>X47 mdaB::ptetR2; urePtetOI</td>
<td>amiE</td>
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<td>ON</td>
</tr>
<tr>
<td>hAD195</td>
<td>X47 mdaB::ptetR2; urePtetOII</td>
<td>amiE</td>
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<td>ON</td>
</tr>
<tr>
<td>hAD196</td>
<td>X47 mdaB::ptetR2; urePtetOIII</td>
<td>amiE</td>
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<td>ON</td>
</tr>
<tr>
<td>hAD197</td>
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<td>amiE</td>
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<td>ON</td>
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<tr>
<td>hAD198</td>
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<td>amiE</td>
<td>OFF</td>
<td>ON</td>
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<td>hAD199</td>
<td>X47 mdaB::ptetR3; urePtetOI</td>
<td>flaA</td>
<td>ON</td>
<td>ON*</td>
</tr>
<tr>
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<td>flaA</td>
<td>ON</td>
<td>ON*</td>
</tr>
<tr>
<td>hAD201</td>
<td>X47 mdaB::ptetR3; urePtetOIII</td>
<td>flaA</td>
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<td>ON*</td>
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<tr>
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<td>flaA</td>
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<td>hAD204</td>
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<td>flaA</td>
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<td>ON</td>
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<td>flaA</td>
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<tr>
<td>hAD208</td>
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<td>flaA</td>
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<td>ON</td>
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<td>hAD209</td>
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<td>OFF</td>
</tr>
<tr>
<td>hAD214</td>
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<td>taTaat</td>
<td>OFF</td>
<td>ON</td>
</tr>
<tr>
<td>hAD215</td>
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<td>taTaat</td>
<td>OFF</td>
<td>ON</td>
</tr>
<tr>
<td>hAD216</td>
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<tr>
<td>hAD217</td>
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<td>taCaat</td>
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</tr>
<tr>
<td>hAD218</td>
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<td>taCaat</td>
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<td>taCaat</td>
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<td>taCaat</td>
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<td>hAD221</td>
<td>X47 mdaB::ptetR7; urePtetOV</td>
<td>taCaat</td>
<td>ON</td>
<td>OFF</td>
</tr>
</tbody>
</table>

*Strains in which urease activity was not significantly reduced when grown in the presence of 50 ng/ml ATc.*
4 Development of the \textit{tet}-system in \textit{H. pylori}

4.1 Introduction

The development of the \textit{tet}-system for generating conditional mutants in \textit{H. pylori} would provide a new genetic tool that could be used to elucidate the different roles that specific genes play in \textit{H. pylori} pathogenesis and in disease progression. The availability of \textit{tet}-conditional \textit{H. pylori} mutants and appropriate animal models will also facilitate the study of gene function at different stages of gastric disease. To date there is no literature evidence of this system being successfully applied in \textit{H. pylori}. Several groups have successfully imported the \textit{tet}-system into other bacteria by introducing one or more modifications to adapt the system to their favourite microorganism so as to study the corresponding disease in animal infection models. Successful transfer of the \textit{Tn10}-derived \textit{tet}-regulatory system from \textit{E. coli} to another organism requires both the expression of the regulator (\textit{TetR}) and the construction of a \textit{tet}-responsive promoter in the target organism.

Both the \textit{TetR} and the \textit{revTetR} based \textit{tet}-systems were selected for the development of tetracycline dependent gene regulation in \textit{H. pylori}. In the latter \textit{revTetR} system, the tetracycline effector is administered only when the target gene needs to be silenced, whereas in the \textit{TetR} system, it must be administered continuously to maintain target gene expression. The \textit{tet} repressors \textit{TetR(BD)} (Schnappinger et al., 1998) and \textit{TetRr1.7} (Scholz et al., 2004) were chosen as they share the same operator (\textit{tetO}) and inducer specificities, allowing for modular compatibility between the two repressor systems. \textit{TetR(BD)} is a chimera of two closely related \textit{TetR} classes, composed of the \textit{TetR(B)} protein DNA-binding domain (aa 1-50) and the \textit{TetR(D)} protein core domain (aa 51-208). \textit{TetR(B)} has a better DNA binding domain that \textit{TetR(D)} and so this chimeric combination has resulted in a \textit{TetR} protein with enhanced stability and better regulatory properties (Schnappinger et al., 1998). \textit{TetRr1.7} is derived from \textit{TetR(BD)}, differing by only three amino acid residues (E15A, L17G and L25V). These mutations are sufficient to reverse the functionality of \textit{TetRr1.7}, making it a repressor in the presence of the effector molecule. Henceforth, the gene encoding \textit{TetR(BD)} will be referred to as \textit{tetR} and its protein product as \textit{TetR}, and the gene encoding \textit{TetRr1.7} will be referred to as \textit{revtetR} and its protein product as rev\textit{TetR}.  

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Chapter 4: Development of the tet-system in *H. pylori*

Previous studies that endeavoured to optimize the tet-system in different host bacteria, demonstrated that constitutive expression of TetRs provided tighter repression. However, the level of TetR expression also affected the induction window of the tet-regulated gene (Ehrt et al., 2005; Kamionka et al., 2005; Lutz and Bujard, 1997). Therefore, in this study, different *H. pylori* promoters will be evaluated for their ability to drive expression of TetRs at levels that will permit efficient regulation of a *H. pylori* tet-responsive promoter.

Translation of the tet-system to new bacterial hosts has often required the development of tet-responsive promoters that work in the target bacterium (Ehrt et al., 2005; Geissendorfer and Hillen, 1990; Rodriguez-Garcia et al., 2005; Whetstine et al., 2009). This has been achieved by replacing non-essential sequences within strong endogenous host promoters with one or more tetO sequences. At the beginning of this study, apart from the vacA (Forsyth and Cover, 1999) and the ureA (Davies et al., 2002) promoters, no other *H. pylori* promoters had been characterized extensively enough to permit such genetic manipulation. A thorough analysis of the ureA promoter conducted by Davies et al. mapped the transcription start point of the ureAB transcript and identified key promoter elements, the -10 region, the extended -10 motif and the -35 region, that are essential for ureA promoter function. Davies et al. also identified the minimal, or core, ureA promoter region which consists of the first 50 bp upstream of the transcriptional start point. Additionally, a single base-pair mutation in the *H. pylori* ureA promoter -10 region (TACAAT) to the consensus *E. coli* sequence (TATAAT) was found to improve the strength of the ureA promoter. This core urease promoter represented an ideal candidate *H. pylori* promoter for the engineering of a tet-responsive promoter. This promoter was small, well characterized and lacked transcription regulatory sequences, such as the NikR and ArsR~P operator sequences, and therefore would not be subject to additional regulation in response to fluctuations in environmental pH and free Ni$^{2+}$ concentration (Figure 4-1) (Contreras et al., 2003; Pflock et al., 2006).
Figure 4-1  Schematic diagram of the ureA promoter

The transcriptional start point is indicated by the black arrow. The white boxes denote the -10 and -35 regions and the light grey oval indicates the operator of the NikR regulator. The black ovals indicate the binding regions of the response regulator ArsR~P. The green bracket indicates the core urease promoter and the blue bracket the region of the 5′ UTR of the ureAB transcript. The uPtetO constructs are composed of the modified core urease promoter and the 5′ UTR. This image has been adapted from (Pflock et al., 2005).

In this study, the stronger, mutated core ureA promoter sequence, containing the consensus E. coli sequence (TATAAT) (Davies et al., 2002), was used to design H. pylori tet-responsive promoters by replacing one or more 17 nt sequences flanking the -35 and -10 regions with tetO sequences. These putative tet-responsive core ureA promoters along with the downstream ureA 5′ untranslated region (UTR) were designated as uPtetO constructs. To test the ability of the uPtetO constructs to drive and regulate gene expression in H. pylori, the green fluorescence protein (GFP) encoded by gfpmut2 (Cormack et al., 1996) was selected as a suitable reporter gene for H. pylori according to a previous report (Heuermann and Haas, 1998).

The mouse adapted H. pylori X47-2AL (X47) strain (Handt et al., 1995; Kleanthous et al., 2001) is a strain that is routinely used to study H. pylori pathogenesis in the C57BL/6J mouse model. X47 is a Type II strain and naturally streptomycin resistant. This strain robustly colonizes several animal hosts, including inbred C57BL/6J, DBA2/2J, FVB/n, CBA/CaH, 129, C3H/HeJ mice (Ondek Pty. Ltd.) and outbred Swiss Webster mice and Mongolian gerbils (Haas, 2010). A drawback to working with strain X47 is its poor plasmid transformability. X47 contains its own endogenous plasmid which has only recently been sequenced by our group, and its properties are currently being studied to better understand plasmid restriction phenotypes and plasmid transfer in strain X47.
This study used strain X47 to develop the \textit{tet}-system for \textit{H. pylori} to enable gene regulation both \textit{in vitro} and \textit{in vivo} in order to study \textit{H. pylori} pathogenesis in animal models. Since a plasmid-based system was not technically feasible in strain X47 at the time of this study, a chromosomally integrated \textit{tet}-system was the only option and candidate loci for insertion of the \textit{tet}-repressor and the inducible \textit{tet}-responsive gene expression cassettes were investigated. Two regions in the \textit{H. pylori} chromosome have previously been described as amenable to gene insertion without affecting colonization, namely the intergenic region between HP0203 and HP0204 (Langford et al., 2006) and the \textit{rdxA} gene (Croxen et al., 2006). Essential components of the \textit{tet}-system, \textit{tetR} and a \textit{tet}-responsive promoter, were integrated into the \textit{H. pylori} X47 chromosome and a reporter gene was used to evaluate if \textit{tet}-regulation had been successfully established in \textit{H. pylori}.

\section{4.2 Aims}

This section of the study addressed the following aims

1. Expression of TetRs in \textit{H. pylori}.
3. Demonstration of tetracycline responsive regulation of reporter gene expression in \textit{H. pylori}. 

4.3 Methods

4.3.1 *H. pylori* growth in the presence of tetracyclines

Individual 5 ml cultures of BHI broth containing increasing amounts of ATc or Dox were inoculated with wild-type X47 bacteria to give a starting OD$_{600}$ = 0.05. Cultures were incubated under microaerobic conditions at 37 °C and 120 rpm. The optical density of the cultures were measured every 4 h for up to 39 h.

4.3.2 GFP reporter assays

4.3.2.1 ATc regulation of GFP expression on plates

Bacteria were plated onto fresh CBA plates with or without 50 ng/ml ATc and after 24 h of incubation were visualized for GFP fluorescence using the LAS 3000 (Fujifilm) (light source: Blue-460nm EPI, filter: GFP510DF10). For Tet-OFF strains, strains containing $ptetR$ (1/3/5/7), bacteria were passaged twice on CBA plates containing ATc prior to the start of the experiment to allow strains sufficient time to repress GFP expression.

4.3.2.2 Disc diffusion assay

Bacteria were plated onto CBA plates, and incubated for 14 h at 37 °C. Blank discs were placed onto the bacterial lawn and inoculated with 30 μl of ATc solution and the bacteria were incubated for another 24 h. GFP expression was visualized using the LAS 3000 (Fujifilm) (light source: Blue-460nm EPI, filter: GFP510DF10).

4.3.2.3 ATc induction of GFP activity

*H. pylori* cultures were grown in 5 ml of BHI media to mid-log phase and then induced for 24 h with 200 ng/ml ATc unless otherwise stated. Bacteria were harvested by centrifugation, washed twice with PBS and resuspended to a density of OD$_{600}$ = 2. Aliquots of the bacterial suspension, 0.1 ml per well, 3 wells per sample, were transferred into black 96-well plates, and fluorescence at 520 nm after excitation at 485 nm was measured using the POLARstar Omega plate reader.
4.3.2.4 ATc induction of GFP expression over time

*H. pylori* cultures were grown to mid-log phase in 10 ml of BHI medium. An aliquot of 2 ml was taken from each culture and used as time 0 and an 8 ml aliquot of fresh media containing 400 ng/ml ATc was added to each culture, to give a final volume of 16 ml and 200 ng/ml ATc. The bacteria were incubated for another 30 h, with aliquots taken from each culture at 2, 4, 8, 16, 24 and 30 h after induction with ATc. Bacteria were harvested by centrifugation, washed twice with PBS and finally resuspended in Tris lysis buffer. The protein concentration of each bacterial cell lysate was measured and 10 μg of protein was loaded in one lane for each sample. Samples were analysed for GFP protein by immunoblotting as described in 2.2.4.2 and 2.2.4.3. PVDF membranes were probed with a rabbit polyclonal antibody against GFP protein (1:2000) followed by incubation with Mouse Anti-rabbit IgG HRP conjugate secondary antibody (1:10000).

4.3.3 Animal Experiments

4.3.3.1 Colonization by recipient loci mutants

Mice were infected with X47 ptetR; uPtetO-GFP strains (Table 3-4, hAD42, hAD58 and hAD60) and sacrificed one week later. The bacterial load in mouse stomachs was evaluated as described in section 2.3.4. Re-isolated bacteria were also visualized for GFP expression to ensure that insertion of uPtetO-GFP and expression of GFP was stable *in vivo*.

4.3.3.2 Tolerance of *H. pylori* X47 to doxycycline *in vivo*

Mice were supplemented with a range of Dox concentrations in their drinking water. Dox supplementation was commenced 24 h prior to infection with wild-type X47 and was maintained for the duration of the experiment. Mice were sacrificed at indicated time points and the bacteria load was evaluated.

4.3.3.3 Regulation of uPtetO *in vivo*

These experiments were designed and conducted in collaboration with Dr. Phebe Verbrugghe. Mice were challenged with conditional GFP expressing strain X47 ptetR4; *trpA*:uPtetO1-GFP (hAD58). Two weeks after infection, animals were supplemented without or with 20 μg/ml ATc in their drinking water for 4 days before they were sacrificed. Stomachs were removed, opened along the greater curvature and gently rinsed with PBS to remove stomach content. Tissue was immediately fixed using 4%
paraformaldehyde in PBS, for 1 h. The fixed tissues were embedded in O.C.T. embedding medium and frozen in liquid nitrogen. Tissue was cut into 16 μm thick sections using a cryostat.

4.3.4 Fluorescence microscopy

Fluorescence imaging of stomach sections was performed by Dr. Phebe Verbrugghe.

In brief, the following protocol was used for all specimens: Cryosections were washed in PBS and incubated with Triton X-100 (0.2 % in PBS) for 5 min to permeabilize the cells. Samples were washed and blocked (4% FCS in PBS) for 20 min, followed by incubation with Rabbit Anti-*Helicobacter* (1:50) and Chicken Anti-GFP (1:2000) for 1 h. Samples were washed again and incubated with Goat Anti-rabbit IgG DyLight 549 (1:500) Goat Anti-chicken IgG Alexa Fluor 488 (1:500) for 30 min. The samples washed again, first with PBS, followed by a wash with water, before incubation with Hoechst (1 μg/ml in PBS) for 10 min to stain cell nuclei. Images were collected using a Nikon Ti-E inverted motorized microscope with Nikon A1S1 spectral detector confocal system running NIS-C Elements software.
4.4 Results

4.4.1 Expression of tet repressors in *H. pylori*

Four different *H. pylori* promoters were selected to drive expression of TetRs in *H. pylori*. The amiE, flaA, and wild-type and mutated core ureA promoters were used to generate different promoter-tetR fusions (ptetR1-7) which were inserted into the *H. pylori* chromosome at the mdaB locus (Figure 4-2).

Expression of TetR and revTetR in X47 mdaB::ptetR strains was detected by immunoblotting. Positive transformants were identified by the presence of a 23 kDa band (Figure 4-3). The expression of TetRs was significantly lower in the X47 mdaB::ptetR strains compared to the *E. coli* positive control (pos) and the signal intensity for X47 strains expressing TetR was greater than for X47 strains expressing revTetR. This was observed for all the promoters used to drive the expression of TetRs (Figure 4-4 A). The expression of TetRs in *E. coli* strains transformed with P_{taTaat} derivatives of pMdaB-ptetR (Table 3-2, eAD1 and eAD2) was analyzed by immunoblotting to determine if this apparent difference in TetR and revTetR expression observed of in *H. pylori* was an artifact produced by the preferential binding of the Anti-TetR antibody to TetR over revTetR. Although TetRs could also be expressed in *E. coli* from all the ptetR constructs (Figure 4-4 B), the P_{taTaat} promoter contained the canonical *E. coli* -10 box (TATAAT), making it the optimal promoter to compare expression of the TetRs from the pMdaB-ptetR plasmids in *E. coli*. The protein concentrations in *E. coli* cell lysates were equalized and titrated out to prevent saturation of the signal. No appreciable difference in signal intensity was observed between the *E. coli* strain expressing TetR and the strain expressing revTetR (Figure 4-4 C).

![Figure 4-2 Schematic diagram of promoter-tetR constructs used in this study](image-url)
Figure 4-3  Screening for expression TetRs in *H. pylori* X47 mdaB::ptetR

Immunodetection of the expression of TetRs by *H. pylori* X47 mdaB::ptetR transformants. The TetRs are a 23 kDa protein, indicated by the black arrows. Blue asterisk denote clones that are positive for TetRs expression. The parent strain, *H. pylori* X47 mdaB::rpsL-cat served as negative control (neg) and *E. coli* transformed with pMdaB-ptetR6 served as positive control (pos).
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**Figure 4-4** Comparison of TetRs expression levels in *H. pylori* and *E. coli*

A. Representative clones for each *H. pylori* X47 mdaB::ptetR strain (1-7) are run together to compare expression of TetRs by each ptetR construct. The TetRs are a 23 kDa protein, indicated by the black arrows. The parent strain, *H. pylori* X47 mdaB::rpsL-cat served as negative control (neg) and *E. coli* harbouring pMdaB-ptetR6 served as positive control (pos). Samples were all run together and developed on the same blot. The lanes have been rearranged for ease of comparison.

B. Immunoblots of TetRs in *E. coli*. Expression of TetRs driven by the four different *H. pylori* promoters are compared in the *E. coli* DH5α host. The TetRs are a 23 kDa protein, indicated by the black arrows. TetRs are expressed in all *E. coli* strains, however the mutant core ureA promoter, P_{taTaat}, drives expression of TetRs in *E. coli* most efficiently.

C. Comparison of TetR and revTetR expression driven by P_{taTaat} in the *E. coli* host. 16 μg (1), 8 μg (1/2) and 4 μg (1/4) of cell lysates from *E. coli* DH5α transformed with either pMdaB-ptetR5 (eAD1) or pMdaB-ptetR6 (eAD2) were loaded. The signal intensity for TetRs protein band is the same for both strains at each sample dilution.
4.4.2 Construction of tetO containing *H. pylori* promoters

To generate a tetracycline responsive promoter for *H. pylori*, one or more tet operator sites (tetO) were introduced into the mutated core ureA promoter sequence while trying to minimize the disruption of key promoter elements. Three main sites were identified for tetO introduction, upstream of the -35 region, between the -35 and -10 regions, and just downstream of the transcriptional start point (Figure 4-5 A). A set of three tet-responsive promoters, uPtetO(1-3), was designed to drive and regulate target gene expression at any recipient locus (Figure 4-5 B).

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**Figure 4-5** Schematic diagram of tetracycline responsive promoters, *uPtetO*(1-3)

A. Nucleotide sequence (partial) of wild-type ureA promoter, *P*<sub>ureA</sub>, and tetracycline responsive *P*<sub>taTaat</sub> derivative, *uPtetO*. The -10 and -35 promoter sequences are underlined and the extended -10 region is shaded in grey. Tet operator (tetO) sequences are indicated by boxes. Arrow indicates transcriptional start point and asterisk indicates the C to T mutation in the -10 region found in the *uPtetO* promoter constructs.

B. Representative diagram of the *uPtetO* constructs. White tetO boxes indicate where the *P*<sub>taTaat</sub> promoter sequence has been replaced with tetO sequences.
Each uPtetO promoter was fused to gfpmut2, to make uPtetO(1-3)-GFP constructs. The strength of the uPtetO promoters was evaluated in the absence of TetRs at three different recipient loci, gltDH, trpA and dapB, using gfpmut2 as a reporter. H. pylori transformed with uPtetO-GFP were grown on CBA plates and GFP fluorescence was detected by visualizing the plates under blue light (Figure 4-6 A). GFP fluorescence was greater in X47 strains harbouring the uPtetO1 promoter compared to strains with the uPtetO2 promoter. GFP fluorescence could not be detected from background bacterial autofluorescence for strains harbouring the uPtetO3 promoter and so this promoter architecture was not evaluated further using the GFP reporter. GFP activity was quantified for H. pylori strains transformed with uPtetO-GFP grown up in BHI media (Figure 4-6 B). After correction for autofluorescence, analysis showed that uPtetO1 was 3- to 4.5-fold stronger than uPtetO2. The strength of each promoter was also influenced by the recipient locus. This locus dependent effect was more evident for uPtetO1 than uPtetO2. X47 strains transformed with uPtetO1-GFP into the trpA locus had 2- and 1.5-fold greater GFP activity compared to strains with uPtetO1-GFP inserted into the gltDH or dapB loci respectively, while strains transformed with uPtetO2-GFP into the trpA locus had only 1.5- and 1.1-fold greater GFP activity compared to strains with uPtetO2-GFP inserted into the gltDH or dapB loci. These results demonstrated that the tetO modified mutant core urease promoters could drive expression of foreign genes from the three different loci tested in H. pylori. The next stage of the study was to test if these promoters could be regulated in a tetracycline dependent manner.

### 4.4.3 Tet-regulation of uPtetO in H. pylori

A panel of X47 ptetR; uPtetO-GFP strains (Table 3-4, hAD38-hAD73) was generated consisting of strains that expressed a tet repressor, either TetR or revTetR, and also expressed GFP, under the control of a tetO containing promoter, from one of three recipient loci. Strains that expressed revTetR were expected to express GFP in the absence of tetracyclines and strains that expressed TetR were expected to only express GFP when grown in the presence of tetracyclines. Strains that lacked TetRs expressed GFP constitutively and were used as control strains.
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Figure 4-6  GFP expression driven by *uPtetO1* and *uPtetO2* in *H. pylori*

A. Bacteria were transformed as indicated, grown on CBA plates and examined under blue light for GFP expression.

- Positions 1 – 4, strains harbouring *uptetO-GFP1*, one tetO site
- Positions 5 – 8, strains harbouring *uptetO-GFP2*, two tetO sites
- Position 9, indicated by arrow, wild-type X47

Panel i) *uPtetO-GFP* inserted into the *trpA* locus, panel ii) *gltDH* locus and panel iii) *dapB* locus. Strains in position 4 of panel ii and position 5 of panel iii are negative for GFP fluorescence.

B. Bacteria were transformed as indicated and grown in BHI to mid-log phase (OD$_{600}$ = 0.5) and GFP activities were determined 24 h later (consistent with experimental conditions used for GFP induction assays). The fluorescence intensity was normalized to the cell density and expressed in relative fluorescence units (RFUs). Data are averages and error bars represent standard deviations. GFP activities of *uPtetO1-GFP* (open bars) and *uPtetO2-GFP* (hatch bars) strains compared to wild-type auto fluorescence (black bar).
4.4.3.1 \textit{H. pylori} growth in the presence of tetracyclines

\textit{H. pylori} is known to be quite sensitive to tetracyclines and consequently tetracyclines are often used in treatment regimens to cure infections that have failed the first round of eradication therapy (Megraud, 2003; Selgrad and Malfertheiner, 2011). Therefore the sensitivity of \textit{H. pylori} strain X47 to Dox and the weak antibiotic analog, ATc, was tested to determine the concentration range that could be used to evaluate the responsiveness of the \textit{uPtetO} promoters to tetracyclines. \textit{H. pylori} was cultured in the presence of ATc or Dox, using concentrations ranging from 0 ng/ml to 1 μg/ml. Growth of X47 was significantly inhibited at 50 ng/ml of Dox and no growth was observed above 100 ng/ml of Dox (Figure 4-7 A). For ATc, growth of X47 started to be significantly inhibited only at 750 ng/ml but was still evident at the highest concentration tested (Figure 4-7 B).

4.4.3.2 Induction of \textit{uPtetO} by ATc

ATc was used as an inducer or repressor, of TetR and revTetR respectively, and GFP activity was used as a reporter to measure the induction and repression potential of \texttt{tet} promoters \textit{uPtetO1} and \textit{uPtetO2}. Based on the observed fluorescence intensities of bacteria growing on CBA plates, addition of 50 ng/ml ATc to blood agar plates resulted in induction of GFP expression in strains expressing TetR (Figure 4-8 B and Figure 4-9 A and D), while, with the exception of \textit{ptetR3} strains, repression of GFP expression was observed in strains expressing revTetR (Figure 4-8 A and Figure 4-9 B and C). In the presence of ATc, GFP expression was only partially repressed in X47 \textit{mdaB::ptetR3; gltDH::urePtetO} strains (hAD52 and hAD53) and could not be repressed in X47 \textit{mdaB::ptetR3; trpA::urePtetO} strains (hAD54 and hAD55) (Figure 4-9 C).

A disc diffusion assay was used to demonstrate induction of GFP expression by ATc. Discs inoculated with ATc were placed onto a bacterial lawn of X47 \textit{mdaB::ptetR4; dapB::uPtetO1-GFP} (Table 3-4, hAD62) (Figure 4-10). After 24 h of incubation, GFP expression was evident only in the area around each disc, where the ATc concentration was sufficient to induce \textit{uPtetO1}. 

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Figure 4-7  Inhibition of \textit{H. pylori} X47 growth by tetracyclines

Growth curves of \textit{H. pylori} X47 grown in BHI without or with different concentrations of (A) Dox or (B) ATc. The optical densities of each culture was recorded every 3 h.
Figure 4-8  *Tet*-regulation of GFP expression in *H. pylori* – Part I

Bacteria were transformed as indicated and grown on plates with or without 50 ng/ml ATc. After 24 h of incubation, plates were examined under blue light for GFP expression.

- Positions 1 – 4, strains harbouring *uptetO1*-GFP, one tetO site
- Positions 5 – 8, strains harbouring *uptetO2*-GFP, two tetO sites
- Position 9, indicated by arrow, wild-type X47

GFP expression from *trpA, gltDH* and *dapB* loci by (A) *ptetR1* strains, and (B) *ptetR2* strains.
Figure 4-9  Tet-regulation of GFP expression in H. pylori – Part II

Bacteria were transformed as indicated and grown on plates with or without 50ng/ml ATc. After 24 h of incubation, plates were examined under blue light for GFP expression.

Positions 1 – 4, strains harbouring uptetO1-GFP, one tetO site
Positions 5 – 8, strains harbouring uptetO2-GFP, two tetO sites
Position 9, indicated by arrow, wild-type X47

GFP expression from trpA, gltDH and dapB loci by (A) ptetR4 strains, (B) ptetR5 strains, (C) ptetR3 strains, and (D) ptetR6 strains.
Figure 4-10  Regulation of GFP expression on plates

Blank discs were inoculated with 30 μl of 1) 0.5 μg/ml, 2) 1.0 μg/ml and 3) 5 μg/ml of ATc and placed onto a bacterial lawn of strain X47 mdaB::ptetR4; dapB::uptetO1-GFP and incubated for 24 h. GFP fluorescence signal appears as a dark signal against the lighter background of the bacterial lawn on the CBA plate. Zone of GFP expression is indicated (white arrows). Discs were removed before the image was taken.

The GFP activity was measured in strains expressing TetR, (ptetR2, ptetR4 and ptetR6), and GFP from either the uPtetO1 promoter or the uPtetO2 promoter. Addition of 200 ng/ml ATc to TetR expressing strains grown in BHI media resulted in an increase in GFP activity after 24 h, however GFP activity did not reach the levels observed in the absence of TetR (Figure 4-11). After 24 h, induction of uPtetO1 was as follows: a 3- to 10-fold increase in GFP activity was measured for strains with ptetR2, a 13- to 80-fold increase in GFP activity was measured for strains with ptetR4, and a 2- to 6-fold increase in GFP activity was measured for strains with ptetR6 (Table 4-1). Induction of uPtetO2 in TetR expressing strains was much weaker; the increase in GFP activity in strains with ptetR2 was 1.3- to 3-fold, 3- to 8-fold for strains with ptetR4, and 1.3-fold for strains with ptetR6. Strain X47 mdaB::ptetR4; trpA::uPtetO1-GFP (hAD58) had the greatest induction range of all the constructs tested, displaying an 80-fold increase in GFP activity upon addition of ATc.
Figure 4-11  Induction of *uPtetO1* and *uPtetO2* in strains with different *ptetR* constructs

Bacteria were transformed as indicated and grown in BHI media to mid-log phase (OD$_{600}$ = 0.5) before 200 ng/ml ATc was added (hatch bars). GFP activities were determined 24 h later. Tet-responsive constructs *uptetO1-GFP* and *uptetO2-GFP* have been transformed into either the (A) *trpA*, (B) *gltDH*, or (C) *dapB* locus. Three independently generated clones were used to measure GFP activities for each construct. All fluorescence measurements were carried out in triplicate. The fluorescence intensity was normalized to the cell density and expressed in RFUs. All fluorescence intensities were corrected for background fluorescence of the bacteria. Data are averages and error bars represent standard deviations.
Table 4-1  Induction and repression of uPtetO in *H. pylori*

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### 4.4.3.3 Induction of uPtetO by ATc in *H. pylori* is dose- and time-dependent

Further characterization of the *tet*-system in *H. pylori* was done by measuring GFP activity after induction with increasing inducer concentrations and by evaluating reporter expression at several different time points. Quantification of GFP activities of *H. pylori* strains grown in the presence of different concentrations of ATc demonstrated that induction of promoters uPtetO1 and uPtetO2 was dependent on ATc concentration and that maximal induction was achieved in all strains with the uPtetO1-GFP construct at a concentration of 100 ng/ml ATc, which is 10-fold below the minimal inhibitory concentration (MIC) of ATc as measured in liquid culture (Figure 4-7 B), while slightly higher concentrations of ATc were required to reach maximal GFP activities for some strains with the uPtetO2-GFP construct (Figure 4-12).
Figure 4-12  Determination of optimal inducer concentration

Comparison of uPtetO1 and uPtetO2 induction in strains with different ptetR constructs. Strains expressing TetR were transformed with tet-responsive constructs uPtetO1-GFP and uPtetO2-GFP as indicated and grown in BHI media to mid-log phase (OD_600 = 0.5) before addition of ATc. GFP activities were determined 24 h later.

(A) P_amiE-tetR (ptetR2)
  i) gltDH::uPtetO1-GFP
  ii) trpA::uPtetO-GFP

(B) P_flmA-tetR (ptetR4)

(C) P_taTaat-tetR (ptetR6)

All fluorescence measurements were carried out in triplicate. The fluorescence intensity was normalized to the cell density, corrected for background fluorescence of the bacteria and expressed in RFUs. Data presented is representative of three independent experiments.
The kinetics of \(uPtetO1\)-GFP induction was analysed in strains expressing TetR by immunoblotting against the GFP protein (Figure 4-13). In \(uPtetO1\)-GFP transformed strains the signal for GFP protein expression increased with ATc incubation time and reached a maximum signal 16 h after the addition of ATc. In \(uPtetO2\)-GFP transformed strains, the difference in GFP protein expression between the repressed and induced states was, as expected, significantly less than for the \(uptetO1\)-GFP strains and in some cases, for \(uPtetO2\) transformed into the \(dapB\) locus (Figure 4-13 F) or for \(ptetR\) strains transformed with \(uPtetO2\) (Figure 4-13 B, D), detection of GFP protein required longer exposure and resulted in detection of additional nonspecific protein signals. Strains expressing TetR under the \(P_{\text{flaA}}\), \(ptetR4\), showed the greatest difference in GFP protein expression between the repressed and induced states for \(uPtetO1\)-GFP and \(uPtetO2\)-GFP constructs inserted at both the \(trpA\) and \(gltDH\) locus.

**4.4.4 Tet-regulation of \(uPtetO\) in vivo**

**4.4.4.1 Testing the colonization ability of recipient strains for conditional gene complementation in vivo**

Once in vitro testing confirmed that the \(tet\)-system was functional in \(H. pylori\), the next step was to determine if chromosomal integration of the \(tet\)-system components affected the ability of X47 to colonize in the C57BL/6J \(H. pylori\) infection model. This would confirm if the four loci used, \(gltDH\), \(trpA\), \(dapB\) and \(mdaB\), were suitable for the stable expression of \(tet\)-responsive genes for future in vivo studies. Mice were gavaged with X47 \(mdaB::ptetR5\) recipient strains transformed with pGltDH-\(uPtetO1\)-GFP, pTrpA-\(uPtetO1\)-GFP or pHdapB-\(uPtetO1\)-GFP (Table 3-4, hAD64, hAD66 and hAD68). These strains constitutively expressed both revTetR and GFP during the course of the experiment. Colonies were successfully re-isolated one week after oral challenge (Figure 4-14), and revTetR and GFP expression were found to be stable after passage through mice.
Figure 4-13  Kinetics of uPtetO induction

Time course of TetR-controlled GFP expression. Bacteria were transformed as indicated and grown in BHI media to mid-log phase (OD$_{600} = 0.5$) before addition of 200 ng/ml of ATc. Aliquots of induced cultures were taken at indicated time points. *H. pylori* lysates were separated on a 10% SDS–PAGE gel. Equal amount of protein (15 μg) was loaded into each lane.

- **Lane 1**, constitutively expressed GFP by X47 lacking TetR (pos)
- **Lane 2**, parent wild-type X47 (neg)
- **Lane 3**, repressed GFP (+ TetR);
- **Lanes 4 – 9**, time course of induction of TetR-controlled GFP by 200 ng/ml ATc.

Induction of uPtetO1 from the A) *trpA*, C) *gltDH* and E) *dapB* locus.
Induction of uPtetO2 from the B) *trpA*, D) *gltDH* and F) *dapB* locus.

Note that for clarity, the contrast has been enhanced individually for each blot, and therefore the signal for GFP protein should not be compared between blots.
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**Figure 4-14 Colonization of C57BL/6J mice by conditional GFP expressing strains**

Three X47 strains, hAD64, hAD66 and hAD68, were tested for their ability to colonize the C57BL/6J mouse model to evaluate if the four chromosomal loci used in this study were suitable for stable gene expression using the *tet*-system for *in vivo* studies. All three input strains expressed revTetR from the *mdaB* locus and expressed GFP, from the *gltDH* locus in strain hAD64, from the *trpA* locus in strain hAD66 or from the *dapB* locus in strain hAD68. Mice were challenged once with a bacterial inoculum and the bacterial load was evaluated one week later. *H. pylori* colonies were re-isolated from all groups challenged with conditional GFP expressing strains. Horizontal bars represent mean bacterial load per group (n = 3), and points plotted represent colonization density for each individual animal. Detection limit was < 20 CFU per stomach (dotted horizontal line). Gastric specimens without *H. pylori* re-isolation are shown as null.

**4.4.4.2 *H. pylori* tolerates low levels of Dox *in vivo***

*In vivo* experiments using *tet*-regulation to study *H. pylori* virulence factors would require supplementing infected animals with tetracycline to regulate gene expression of conditional *H. pylori* mutants. A method for tetracycline administration which was suitable to experiments that were long in duration and that used large numbers of animals was required. The administration method used by Gandotra and co-workers to study *M. tuberculosis* persistence seemed most appropriate (Gandotra et al., 2007), however unlike *H. pylori* strain X47, the *M. tuberculosis* strain used by Gandotra et al. was highly resistant to tetracycline. Therefore a series of infection experiments using wild-type X47 was conducted to identify the maximum dose of Dox which would be tolerated by *H. pylori in vivo*. C57BL/6J mice were challenged with wild-type strain X47 and their drinking water was supplemented with a range of different Dox concentrations. One week after challenge, the bacterial load was significantly reduced at a Dox concentration of 100 μg/ml and bacteria could not be re-isolated from mice supplemented with 1000 μg/ml of Dox, the same dose used by Gandotra *et al.* to regulated gene expression in *M. tuberculosis* (Figure 4-15 A). At Dox concentrations...
above 1 μg/ml, the bacterial load decreased by two logs, however at the longer infection times of two and four weeks, the bacterial load in mice supplemented with 5 μg/ml Dox was similar to that of untreated animals (Figure 4-15 B).

**Figure 4-15  *H. pylori* X47 tolerance to Dox in vivo**

A. Mice were challenged with wild-type strain X47 and supplemented with a range of Dox concentrations in their drinking water. Bacterial load was evaluated one week after challenge. The data presented is from three separate experiments. Horizontal bars represent mean bacterial load per group (n = 3) and points plotted represent colonization density for each individual animal. The detection limit was < 20 CFU per stomach (dotted horizontal line). Gastric specimens without *H. pylori* re-isolation are shown as null.

B. Mice were challenged with wild-type X47 and supplemented with 5 μg/ml Dox in their drinking water. Bacterial load was evaluated at two and four weeks after challenge. Horizontal bars represent mean bacterial load per group (n = 6).
4.4.4.3 Tet-regulated GFP expression in vivo

With the optimal inducer concentration in hand, *in vivo* tet-regulation of *uPtetO* was investigated in collaboration with Dr. Phebe Verbrugghe. Mice were challenged with strain X47 *mdaB::ptetR4; trpA::urePtetO1-GFP* (hAD58), which only expressed GFP in the presence of tetracyclines. Two weeks after challenge, one group of animals was started on tetracycline supplementation while another group was maintained on standard drinking water. Confocal fluorescence imaging of infected mouse stomachs detected significantly greater amounts of GFP protein in the stomachs of mice that received tetracycline compared to the stomachs of infected mice that remained untreated. This data demonstrated that tetracycline supplementation could regulate *uPtetO1* in strain hAD58 during infection *in vivo*. 
Figure 4-16  Induction of GFP expression in *H. pylori* during infection

Stomach sections from mice infected with conditional GFP expressing *H. pylori* strain hAD58. Two weeks after infection, one group of mice received 20 μg/ml ATc for 4 d in their drinking water (bottom), whereas the control groups were kept without ATc supplement (middle). The top row depicts sections stained with secondary antibodies only. The left column depicts stomach tissues stained with antibody against *H. pylori*, the middle column depicts tissue stained with antibody against GFP and the right columns shows cell nuclei counterstained with Hoechst (blue), merged with Anti-*H. pylori* (red) and Anti-GFP (green) stain.
4.5 Discussion

This section of the study aimed to 1) express tet repressor proteins in *H. pylori*,
2) develop tet responsive *H. pylori* promoters, and use them together to 3) regulate
reporter gene expression in a tetracycline dependent manner.

4.5.1 Expressing TetRs in *H. pylori*

Several studies utilizing the tet-system concluded that constitutive expression of TetRs
was more favourable for tight repression of strong promoters than the original
autoregulated expression approach derived from the Tn10 Tc resistance determinant
(Ehrt et al., 2005; Kamionka et al., 2005; Lutz and Bujard, 1997). Based on these
conclusions, the approach involving constitutive expression of TetRs was chosen for
this study. Four *H. pylori* promoters were selected to provide a range of TetR expression
so as to permit fine-tuning of gene induction under different growth conditions. The
PamiE promoter was chosen as it was successfully used to drive lac repressor expression
in a plasmid-based lacP8 conditional expression system (Boneca et al., 2008) while PflnA
was chosen as it had been previously used as a strong promoter to drive GFP expression
in *H. pylori* (Heuermann and Haas, 1998; Li, 2009). The core urease promoters were
also selected for this study because they lack the NikR and ArsR regulatory operators
and therefore should not be subject to changes in the environment, making them truly
constitutive promoters. Furthermore using both PtaCaat and the stronger PtaTaat promoter
would ensure that at least one of the four promoters selected in this study would differ
in strength from the others.

Since the expression of foreign proteins is not as well established in *H. pylori* as it is in
*E. coli*, the coding sequences of tetR and revtetR were joined to the different promoters
and their corresponding 5′ UTR regions by PCR to generate ptetR(1-8). This strategy
would preserve the *H. pylori* promoters as functional units and avoid introducing any
changes to the 5′ UTRs that could have adverse effects on protein translation (Knaus
and Bujard, 1990) and therefore rule it out as a contributing factor if problems with
TetRs expression arose. Transformation of these constructs into the rdxA locus of
*H. pylori* X47 proved unsuccessful. This meant that either the tet repressor was toxic to
*H. pylori*, or alternatively, that transformation in the rdxA locus in strain X47 was not
very efficient. The latter proved to be the case as changing the integration locus to the mdaB region resulted in transformants harbouring the ptetR constructs and >85% of those screened expressed the tet repressor proteins. Interestingly, in H. pylori, revTetR was expressed in lower quantities than TetR, regardless of the promoter used to drive its expression. This phenomenon was not observed in the E. coli DH5α host, indicating that in H. pylori the three amino acid mutations in revTetR significantly decreased its stability, resulting in lower steady state levels of revTetR compared to TetR. The same phenomenon, observed in both B. subtilis (Kamionka et al., 2005) and in M. tuberculosis (Guo et al., 2007), led to incomplete repression of the reporter gene by revTetR. Kamionka et al., using the same tetR alleles described in this study, overcame the repression problem in B. subtilis by using a stronger promoter to increase revTetR expression and consequently achieved gene silencing that matched TetR. Guo et al., using alleles tetR(B) and tetR(BD) r1.7, employed the same strategy and also obtained improved revTetR repression. Later work by the same group attributed this disparity in repression between TetR and revTetR to the TetR(BD) chimera being less stable in Mycobacteria than TetR(B) (Klotzsche et al., 2009). Therefore to achieve additional improvement in repression by revTetR in Mycobacteria, gene codon usage of revtetR was adapted for M. tuberculosis to increase revTetR steady state levels, and then for further improvements, the revTetR phenotype was re-engineered in the codon optimized tetR(B) allele through directed mutagenesis to achieve both increased protein stability and improved activity. If the reduced levels of revTetR expression observed in H. pylori prove to be too low to repress tet-regulated promoters, the above examples present some options for improving revTetR expression in H. pylori.

4.5.2 Development of H. pylori tet-regulated promoters

The mutated core ureA promoter, P_{uTaT}, was selected for the development of a tet-responsive promoter in H. pylori. P_{uTaT} was ideal because it is a strong promoter, which would permit a large regulatory window, it lacks the ureA promoter transcription regulatory domains and would therefore be minimally influenced by environmental changes, and finally the key promoter elements have been previously identified and therefore disruptions to these sequences could be minimized when selecting promoter sequences to be replaced with tetO sites. One tetO site replaced the sequence near the transcriptional start point (TSP) of P_{uTaT} without altering the base-pairs upstream of the TSP. Another tetO site was placed between the -10 and -35 region, replacing the 17 nt
sequence upstream of the important extended -10 motif and avoiding mutations in the -35 region, and a final tetO site replaced sequences upstream of the -35 region. There are four main tet-promoter architectures described in the literature; one tetO site inserted between the -10 and -35 region (Geissendorfer and Hillen, 1990), two tetO sequences flanking the -10 region (Ehrt et al., 2005) or flanking the -35 region (Lutz and Bujard, 1997) and three tetO sequences in all three positions (Rodriguez-Garcia et al., 2005). Recently, a fifth tet-promoter architecture has been described with one tetO sequence inserted downstream of the transcriptional start point (Whetstine et al., 2009). In this section of the study, three of the five promoter architectures were investigated for generating H. pylori tet-responsive promoters, uPtetO (Figure 4-5 B).

The uPtetO promoters were constructed using multiple long forward primers which facilitated introduction of the tetO sites and several restriction sites for easy replacement of either the promoter or target gene in future studies. PCR construction of the uPtetO-GFP fusion constructs required several attempts. The long forward primers contained repetitive palindromic sequences (tetO) which likely led to primer annealing and the formation of secondary structures. To inhibit secondary structure formation, 5% DMSO was added to Pwo DNA polymerase containing PCRs (Sambrook and Russell, 2001). This strategy successfully amplified the DNA constructs, however sequencing of the final uPtetO constructs identified single and double base-pair deletions in the tetO sequences. An alternative strategy, using AccuPrime™ DNA polymerase mix coupled with optimization of annealing temperatures, generated the correct uPtetO constructs. The AccuPrime™ mix contains accessory proteins that enhance specific primer-template hybridization and thus made amplification of the DNA template possible without the need for other PCR additives like DMSO. However, in view of the rapid progress in DNA synthesis and its affordability since this work was started, future optimisation of the tet responsive promoter should rely on in silico design and outsourcing of DNA synthesis rather than annealing overlapping oligonucleotides and cloning.

Once the uPtetO-GFP constructs were made they had to be inserted into the H. pylori chromosome. The two genomic regions previously reported as neutral for gene insertion were very difficult to work with in strain X47. Insertion of the rpsL-cat counterselection cassette at these loci required several attempts which eventually yielded some transformants however replacement of the cassette with DNA of interest at these loci
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failed to yield the desired mutants in strain X47. Use of the intergenic region between HP0203 and HP0204 was demonstrated to work well in strain 26695 but with significantly less efficiency in strains SS1, J99 and 43504 (Langford et al., 2006), while the rdxA locus has been used in several strains including strain X47 (Croxen et al., 2006; Cullen et al., 2011; Smeets et al., 2000). Since, in this study, the intergenic region between HP0203 and HP0204 and the rdxA gene were found to be unsuitable for routine genetic manipulation in strain X47, and the mdaB locus was occupied by the ptetR constructs, another region in the X47 chromosome had to be identified for integration of uPtetO-GFP. Chromosome-based tet-regulation in H. pylori in this and future studies will routinely require manipulation of the genome, therefore several genomic regions were investigated as recipient loci for genetic manipulation. The loci investigated in this study, gltDH, trpA and dapB proved to be very efficient within strain X47. Cloning vectors made for two loci, the gltDH and trpA locus, were made in pBluescript so that high yields of plasmid DNA could be isolated from a small E. coli culture and used for multiple transformations. The cloning vector for the third locus, dapB, was made in a low copy vector pHSG576 to permit cloning and manipulation of H. pylori genes that are toxic to E. coli. In this study transformation of X47 in all three loci worked efficiently.

Comparison of the GFP fluorescence intensities of strains transformed with uPtetO-GFP constructs demonstrated that expression decreased with increasing number of tetO sites, and furthermore, was influenced by the genomic locus into which the constructs were transformed. Replacement of promoter sequences between -10 and -35 regions with a second tetO site in uPtetO2 significantly affected its activity compared to uPtetO1. A recent analysis of ~2000 transcription start signals identified a periodic AT-rich signal upstream of position -14 as a moderately conserved feature in H. pylori promoters (Sharma et al., 2010). Therefore the decreased activity of uPtetO2 can be attributed to the decrease in AT content with tetO sequence replacement. Interestingly, however, this region is the prime location for tetO in published tet-promoter architectures. Therefore, analysis of additional tet-promoter architectures (e.g., a single tetO between -10 and -35 region) should be done in H. pylori to confirm the observations in this study (This is discussed further in section 6.5.1). The addition of a third tetO site in uPtetO3 reduced GFP activity even further to near background autofluorescence levels and so this promoter architecture was not evaluated further using the GFP reporter.
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The locus dependent effect on $uPtetO$ activity was also an interesting observation. Variations in gene expression due to chromosomal positioning is well documented in other bacteria, such as *E. coli* (Cebolla et al., 1997), *Salmonella typhimurium* (Schmid and Roth, 1987) and *Lactococcus lactis* (Thompson and Gasson, 2001), and has been attributed to the operative increase in gene dosage associated with regions close to oriC.

This explanation could be considered for *H. pylori*, as $uPtetO$ activity was greatest at the *trpA* locus, the locus nearest to oriC (~300 kb). However, unlike *E. coli*, *H. pylori* is a slow replicating organism and thus it is likely not to have as many initiated replicating chromosomes as *E. coli*. Therefore an operative increase in gene dosage would not be as significant in *H. pylori* and this is supported by the $uPtetO$ activity data (Figure 4-11) which was the lowest when inserted at the *gltDH* locus despite being closer to oriC (~400 kb) than when inserted at the *dapB* locus (~540 kb). Nevertheless, the positional effects on $uPtetO$ activity provide an additional mechanism by which gene expression in conditional mutants can be adjusted to match the levels of gene expression in wild-type strains.

4.5.3 Tet-regulation of $uPtetO$ in *H. pylori*

Comparative analysis of strains transformed with $uPtetO$-GFP and different *ptetR* constructs demonstrated that the $uPtetO$ constructs could be regulated in *H. pylori* in a tetracycline dependent manner both on solid media and in liquid culture. Plate induction demonstrated that both TetR and revTetR could be used to regulate $uPtetO$. Despite the low steady state levels of revTetR protein, expression from some *ptetR* constructs was sufficiently high to reduce GFP expression on CBA plates. The mixed repression results for strains expressing revtetR under P flaA seems to indicate that the level of revTetR expression was the lowest in these strains, compared to strains transformed with the other promoter-revtetR constructs, as transcription from $uPtetO$ inserted in the *gltDH* locus could be repressed while transcription from $uPtetO$ inserted at the *trpA* locus could not. This may support the theory that operative gene dosage is higher at the *trpA* locus in *H. pylori* as the amount of revTetR in *ptetR*3 transformed strains is insufficient to silence the greater number of $uPtetO$ constructs in strains with $uPtetO$ at *trpA* compared to strains with $uPtetO$ at *gltDH*. At this stage, the study focused on TetR mediated tet-regulation in *H. pylori*. Analysis of revTetR mediated tet-regulation in *H. pylori* will be the subject of future studies.
A more extensive analysis of TetR expressing strains induced in liquid media demonstrated that GFP activity could be titrated by varying either the ATc concentration or the amount of TetR protein expressed by H. pylori. Using P_{taTaat} to drive tetR expression resulted in the highest steady state levels of TetR protein, followed by P_{taCaat}, P_{amiE} and finally by P_{taA} which produced the lowest amount of TetR. These differences as detected by immunoblotting were not very large however they had significant effects on uPtetO1 regulation. Lower amounts of TetR protein makes the tet-system more sensitive to the presence of ATc and results in faster induction responses (Hillen and Berens, 1994), which was observed in strains transformed with ptetR4 (P_{taA}-tetR). These strains had the greatest range in GFP expression, responding to as little as 5 ng/ml of ATc and reaching maximal levels of GFP earlier, compared to strains that were transformed with ptetR2 or ptetR6. Conversely, induction of uPtetO was both lower and slower in strains transformed with ptetR6, strains expressing the greatest amount of TetR, however repression of uPtetO in the absence of ATc was not more efficient. One complication of the study was due to the background signal (bacterial autofluorescence) which made it difficult to measure low amounts of GFP activity. To make more accurate comparisons of repressed uPtetO activity in different strains, a reporter system with shorter protein half-life and more specific activity, such as an enzymatic activity like that of lacZ or luciferase, would be more appropriate. The ptetR dependent effect on uPtetO2 regulation was not as pronounced in uPtetO2-GFP transformed strains due to the smaller range in expression between induced and repressed states. Addition of the second tetO site did not result in measurable improvement in repression of uPtetO2 compared to uptetO1. This may again be due to the nature of the reporter used in this study and should be investigated in future studies using a more sensitive reporter system.

The results obtained from in vitro induction experiments demonstrated that tet-regulated gene expression in H. pylori works and therefore could serve as a useful tool to study H. pylori virulence factors. Therefore the potential for tet-regulation of H. pylori genes during infection was investigated. Infection models for other bacteria have used a variety of administration methods. Studies in S. aureus and Y. pestis administered ATc once daily, either orally or by injection, for periods ranging from 3 to 7 days (Ji et al., 1999; Ji et al., 2001; Lathem et al., 2007). The model for persistent M. tuberculosis infection extends up to 16 weeks (Gandotra et al., 2007) and gene expression was regulated in this model by supplementing mouse drinking water with Dox. Since the
bacterial load of X47 in the C57BL/6J mouse infection model reaches a plateau only after 2 weeks of infection (Ondek Pty. Ltd., unpublished data), self-administration of tetracyclines by the animals was considered the most practical administration method for *H. pylori* infection experiments. Compared to intraperitoneal or tail vain injection, administration of tetracyclines in the drinking water would provide more regular dosing and, over all, would be significantly less discomforting for the animals and would reduce the work load required for experiments that often range between 1 and 4 weeks in length. An *in vivo* dose response curve confirmed *in vitro* data that strain X47 was quite sensitive to Dox. The maximum dose tolerated by X47 was 10-fold below the standard Dox concentration used to regulate gene expression in transgenic mice and 5-fold lower than the administered dose in the *M. tuberculosis* infection model. However, *M. tuberculosis* is significantly more resistant to tetracyclines than *H. pylori* and the site of infection is far from the source of administration. Therefore the administrative dose likely had to be very high in the *M. tuberculosis* infection model in order to reach the bacteria in the lungs at concentrations that could overcome the action of multidrug efflux pumps and induce gene expression. *H. pylori*, on the other hand, is situated in the stomach and does not possess resistance mechanisms that will decrease the intracellular concentrations of tetracyclines (Megraud, 2003). Therefore it is likely that the administrative dose of Dox supplement will not need to be very high in order to affect gene expression. Furthermore at such low concentrations, despite the increased cost, administration of the less toxic and more potent ATc analog becomes a feasible option for small experiments.

Secondary mutations that affect colonization may have arisen during the multiple genetic engineering steps to construct the final X47 recombinant strains. To rule this out and to assess the expression stability of the GFP reporter gene under the *tet*-regulation, the ability of X47 *ptetR; uPtetO-GFP* strains to colonize C57BL/6J mice was tested. Gratifyingly, GFP expression was stable in all three loci and the strains retained their ability to colonize C57BL/6J mice even after four transformations and five freeze-thaw cycles. This result validates the approach used in this study to construct a chromosomally based *tet*-inducible system in *H. pylori*. Finally *tet*-regulation of conditional GFP expressing strains was tested *in vivo*. The conditional strains made in this study expressed relatively low amounts of GFP protein upon induction, resulting in fluorescence signals which were not much greater than the background autofluorescence from mouse stomach tissue and the GFP fluorescence signal also faded very quickly.
Consequently it was necessary to use an antibody against GFP in the confocal microscopy studies to image GFP expression by *H. pylori* in stomach tissue. These studies showed that the conditional X47 strains expressed significant amounts of GFP protein *in vivo* only when their hosts received ATc supplement, demonstrating that the low dose of tetracycline used was sufficient to regulate gene expression in *H. pylori*. Interestingly, not all the bacteria in the stomach seem to be expressing GFP. This data may reflect that the bacteria are present in different metabolic states, bacillary vs. coccoid, in the stomach and therefore unable to respond to tetracycline regulation (Chan et al., 1994; Kusters et al., 1997). Alternatively, since the antibody used to detect *H. pylori* is a polyclonal and therefore recognizes *H. pylori* antigens and not only live cells, the greater anti-*Helicobacter* signal may be due to not only to intact *H. pylori* that contain GFP but also due to the outer membrane vesicles produced by *H. pylori* (Fiocca et al., 1999; Keenan et al., 2000) and due to the antigens released by specific export or bacterial cell lysis, which could remain in the stomach by adhering to the gastric mucosa and to live *H. pylori* cells (Marcus and Scott, 2001; Phadnis et al., 1996). Nevertheless, the confocal data clearly demonstrated that gene expression from the tet-responsive uPtetO1 promoter could indeed be regulated *in vivo* during the persistence stage of infection using low levels of tetracycline supplementation.

In summary, tet-regulation has been demonstrated to work in *H. pylori* using the reporter gene, GFP. The use of tetracycline dependent gene regulation has not been previously reported for *H. pylori* or for any other members of the ε-proteobacteria. This study describes different uPtetO and ptetR constructs that can be combined with new chromosome positions that are amenable to neutral gene insertion, to provide three different levels of regulation for modulating and fine tuning gene expression in conditional mutants. Tet-regulated GFP expression both *in vitro* and *in vivo* has established that tet-regulated gene expression in *H. pylori* is possible and shown that further experimentation with the tet-system as a genetic tool to study *H. pylori* virulence factors is warranted.
5 Tet-regulation of CGT in *H. pylori*

5.1 Introduction

Like most bacteria, *H. pylori* is unable to synthesize its own cholesterol (Alm et al., 1999; Tomb et al., 1997). However, when grown in cholesterol supplemented medium, up to 25% of the bacterium’s total lipids consist of cholesterol based steryl glycoside derivatives (Haque et al., 1996; Hirai et al., 1995). Steryl glycosides are membrane lipids composed of a sterol covalently linked to one or more carbohydrate residues and are synthesized by all vascular plants, most fungi and algae, and have also been identified in some animals and bacteria (Grille et al., 2010). The glycosidic bond between the sugar moiety and the 3β-hydroxy group at the C3-atom of the sterol can adopt one of two configurations, α or β, with the β-configuration being the most abundantly found (Figure 5-1 A). Helicobacters are one of the few organisms that contain steryl glycosides with the sugar attached to cholesterol in the unusual α-configuration (Figure 5-1 B).

![Figure 5-1 Structure of steryl glycosides](image)

The glycosidic bond between the sugar moiety and the 3β-hydroxy group at the C3-atom of the steryl can adopt (A) the more common β-configuration, or (B) the rarer α-configuration.

Cholesterol (Ch) is not essential for *H. pylori* growth, but the bacterium grows very poorly without it and so cholesterol is an excellent growth enhancer (Testerman et al., 2001). *H. pylori* is able to sequester cholesterol from the medium or even directly from epithelial cells (Wunder et al., 2006) but the mechanism for cholesterol uptake remains unknown. A majority of the cholesterol (> 90%) is converted to one of three different glucosylated cholesterol derivatives: cholesteryl α-D-glucopyranoside (αCG),
cholesteryl 6-O-tetradecanoyl-α-D-glucopyranoside (αCAG) and cholesteryl 6-O-phosphatidyl-α-D-glucopyranoside (αCPG) (Figure 5-2) (Haque et al., 1996; Haque et al., 1995). The enzyme cholesterol α-glucosyltransferase (CGT), attaches glucose to cholesterol to form αCG (Lebrun et al., 2006; Lee et al., 2006). The other two glucosylated cholesterol derivatives are made from αCG by the addition of an acyl or phosphatidyl moiety to the C6 atom of the glucose residue of αCG to form αCAG and αCPG respectively. The enzymes that biosynthesize αCAG and αCPG have yet to be identified. The αCPG steryl glucoside is very unique and thus far, phosphate containing steryl glycosides have only been identified in Helicobacter (Grille et al., 2010).

The cholesteryl α-glucosides of H. pylori are thought to be important for maintaining membrane stability and for immune evasion. The relative proportions of the cholesterol derivatives can vary depending on two main factors. The morphological state of the bacterium is critical as spiral shaped H. pylori cells have almost equal proportions of αCG and αCAG and very little of the αCPG derivative, while in coccoid H. pylori cells, induced by stressful culture conditions, the majority of the αCG derivative is converted to αCAG and αCPG (Shimomura et al., 2004). The relative proportions of each cholesterol derivative are also linked to the lysophospholipid content of the H. pylori membrane (Tannaes and Bukholm, 2005). Lysophospholipids are biosynthesized from phospholipids by enzymatic removal of an acyl chain. Under acidic growth conditions, the lysophospholipid content in H. pylori lipid membranes becomes elevated to unusually high levels (50% of the total phospholipids) compared to the very low levels found in most bacteria (Tannaes et al., 2001; Tannaes et al., 2000). H. pylori strains with high levels of lysophospholipids are more virulent as they are able to release more urease and VacA, and they adhere to and invade host cells more efficiently (Bukholm et al., 1997). These strains are also associated with an increased risk of peptic ulcer disease (Tannaes et al., 2005). However, high levels of lysophospholipids are usually toxic to bacterial cells and it has been proposed that H. pylori counteracts the membrane destabilizing effects of high lysophospholipid levels by increasing the proportion of αCAG, the more rigid cholesteryl glucoside derivative, to restore and maintain membrane stability (Tannaes and Bukholm, 2005).
Figure 5-2 Structures of cholesteryl α-glucosides found in *H. pylori*

The three main cholesteryl α-glucosides found in *H. pylori* are: αCG, αCAG and αCPG. R1 and R2 represent the alkyl chains of 14:0, 18:1, and 19:0 fatty acids. Note a derivative of αCPG is known as lyso-αCPG where the R1-acyl chain is not present. (Adapted from (Lebrun et al., 2006))
Cholesteryl α-glucosides have also been shown to have additional biological functions pertaining to host immune modulation and immune evasion. In the mouse model, compared to wild-type H. pylori, mutants lacking cholesteryl α-glucosides were more readily phagocytosed by macrophages which led to significantly enhanced specific T cell activation (Wunder et al., 2006). These effects could be counteracted when the H. pylori mutants were fed with αCG, but not βCG, indicating that the spatial orientation of the glucose is very important to the immune evasion properties of cholesteryl α-glucosides. Furthermore, unlike wild-type, H. pylori mutants deficient in cholesteryl α-glucosides could not be detected at 12, 24 and 48 h after infection, as they were quickly cleared from mouse gastric tissue, demonstrating that cholesteryl α-glucosides were also required for robust H. pylori infection in vivo (Wunder et al., 2006). Finally, recent work using human derived T cells demonstrated that cholesteryl α-glucosides were required for H. pylori to directly inhibit CD4+ T cell proliferation (Beigier-Bompadre et al., 2011). Overall, these data suggest that the immune evasive function of cholesteryl α-glucosides from H. pylori may constitute a new mechanism of pathogen persistence. The details of this mechanism are unknown, but it has been proposed that the molecular function of αCGs are to interfere with receptor-mediated uptake of H. pylori by phagocytes and, for the first few bacteria that are successfully engulfed, bacterial αCGs are incorporated into the total cholesterol pool inside the phagocyte, thereby interfering with further phagocytosis and downstream signaling of membrane receptors. (Grille et al., 2010)

Studying the biological functions of cholesteryl α-glucosides in H. pylori only became possible when the enzyme that makes the first cholesteryl α-glucoside, αCG, was identified and cloned. This enzyme, cholesterol α-glucosyltransferase (CGT), is encoded by the HP0421 ORF (Lebrun et al., 2006; Lee et al., 2006). CGT transfers glucose, using uridine 5′-diphosphoglucose (UDP-glucose) as a substrate, to the 3-β position of cholesterol, resulting in the formation of αCG (Figure 5-3) (Lebrun et al., 2006). Based on the Carbohydrate-Active enzymes (CAZy) classification system, CGT belongs to family 4 of the glycosyltransferases (GT4) whose catalytic mechanisms lead to the retention of the anomic configuration of the sugar moiety (Cantarel et al., 2009). CGT is a soluble protein that is found to be both in the cytoplasm and associated with the bacterial membrane, most likely at the cytoplasmic face of the inner membrane, however only membrane associated CGT is catalytically active (Hoshino et al., 2011; Lebrun et al., 2006).
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**Figure 5-3  Enzymatic reaction catalyzed by CGT**

The mechanism of CGT regulation remains largely unknown, although there is evidence to suggest that its expression is controlled at a transcriptional level. The gene encoding the enzyme, *cgt* (HP0421), is flanked by the upstream gene HP0420, encoding a hypothetical protein, and the downstream metabolic gene of arginine decarboxylase, HP0422 (Figure 5-4). *cgt* is transcribed as part of the HP0417-HP0422 operon, and also as part of the HP0420-HP0422 suboperon, recently identified by characterization of the *H. pylori* primary transcriptome (Sharma et al., 2010). Transcription of both these operons was found to increase more than 2-fold under acidic stress (pH 5.2), while transcription of the HP0420-HP0422 suboperon decreased more than 10-fold when the bacteria were grown in contact with responsive gastric epithelial cells (Sharma et al., 2010). Furthermore, an antisense RNA transcript against *cgt* was also identified, suggesting a potential mechanism for specific regulation of *cgt* over the other co-transcribed genes.

**Figure 5-4  Schematic diagram of the cgt genomic locus**

The ORFs of the HP0417-HP0422 operon and HP0420-HP0422 suboperon are depicted. The size of each ORF is indicated below (bp). The transcriptional start points are indicated by the red arrows. The antisense RNA transcribed from this locus is indicated by a blue arrow.
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To broaden our understanding of the biological functions of cholesteryl α-glucosides, especially in the role of immune evasion and persistence *in vivo*, it would be of interest to generate conditional CGT mutants using the *tet*-system adapted to *H. pylori* (Chapter 4). As *cgt* is transcribed as part of an operon, a *tet*-promoter cannot be placed at the native *cgt* locus to regulate its expression since it would also regulate the expression of the downstream HP0422 gene. The HP0421 ORF was therefore removed to maintain the integrity of the HP0417-HP0422 operon and the *cgt* mutant was genetically complemented by introducing *cgt* under the control of the *uPtetO* *tet* promoters at one of the suitable recipient loci identified in this work (Chapter 4). To study the role of CGT and cholesteryl α-glucosides in persistence in the C57BL/6J mouse model, the conditional CGT mutant was made using the mouse colonizing *H. pylori* strain X47 (Chapter 4.1).

5.2 Aims

This section of the study addressed the following aims.

1. Generation of a *cgt* mutant in *H. pylori* strain X47.
2. Complementation of the X47 *cgt* mutant using the *uPtetO-CGT* cassette.
3. Generation of a conditional, tetracycline responsive, X47 *cgt* mutant.
4. Characterization of changes in host response and infection load upon restriction of *cgt* expression *in vivo*. 
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5.3 Methods

5.3.1 *In vitro* analysis of CGT activity in *H. pylori*

5.3.1.1 TLC analysis of total cholesteryl glucosides.

Lipids were extracted based on methods previously described (Lebrun et al., 2006; Wunder et al., 2006). *H. pylori* cells were grown in BB, BHI or HI media for 24 h as described in section 2.2.1.2. Cells were harvested by centrifugation (4 °C, 10 min, 4,000 g) and the cell pellet was washed twice with PBS. Lipids were extracted from the cell pellet with chloroform/methanol 1:2 (v/v) and then chloroform/methanol 2:1 (v/v). The extracts were combined and the organic phase was evaporated. The residue was weighed and dissolved in a 1:1 chloroform/methanol solution (v/v 100 μl/ mg of residue). The extracted lipids were separated by thin-layer chromatography (TLC) on silica gel 60 plates (Merck) using methanol/chloroform 15:85 (v/v) as an eluant and were developed with a 13 mM α-naphthol/sulfuric acid/methanol 3:97 (v/v) solution at 160 °C. Standards of αCG and αCAG were synthesised by Dr. Keith Stubbs (Perth, Australia).

5.3.1.2 TLC analysis of cholesteryl glucosides using NBD-cholesterol

*H. pylori* cells were grown in liquid culture for 24 h in the presence of 1 μg/ml 25-NBD-cholesterol. Lipids were extracted and separated by TLC as described in section 5.3.1.1. NBD-cholesterol derivatives were visualized using a Typhoon™ phosphorimager (direct excitation 457/488 nm) or the LAS 3000 (Fujifilm) (light source: Blue-460nm EPI, filter: GFP510DF10).

5.3.2 Immunodetection of CGT-HA expression

PCR positive X47 *HP0421-HA* clones and strains harbouring *uPtetO-CGT-HA* (Table 3-5) were analysed for CGT expression by immune detection. Bacteria growing on CBA plates or in HI media were collected and prepared as described (2.2.4.1). For each sample, equal amounts of protein were loaded and separated on a 10% polyacrylamide gel and then electroblotted onto a PVDF membrane (2.2.4.2). Membranes were first probed with Anti-HA antibody (1:1000) and then probed with Rabbit Anti-mouse IgG HRP conjugate secondary antibody (1:10000) (2.2.4.3).
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5.3.3 Animal Experiments

5.3.3.1 Colonization test of first generation cgt mutant strains.
C57BL/6J mice, n = 3 per group, were orally challenged with one dose of 10^9 CFU of first generation cgt mutants, X47 HP0421::rpsL-cat-1 and four independent clones of X47 ΔHP0421. Mice were sacrificed one week after oral challenge and the bacterial load was evaluated as described in section 2.3.4.

5.3.3.2 Colonization test of uPtetO-CGT complemented first generation cgt mutant strains.
C57BL/6J mice, n = 3 per group, were orally challenged with one dose of 10^9 CFU of uPtetO-CGT complemented first generation HP0421 mutant strains, X47 ΔHP0421; gltDH::uPtetO1-CGT, X47 ΔHP0421; trpA::uPtetO1-CGT, X47 ΔHP0421; trpA::uPtetO2-CGT, X47 ΔHP0421; dapB::uPtetO1-CGT, X47 ΔHP0421; dapB::uPtetO2-CGT (Table 3-2, hAD79-hAD81, hAD83 and hAD84). Mice were sacrificed two weeks after oral challenge and the bacteria load was evaluated.

5.3.3.3 Colonization test of second generation cgt mutant strains.
C57BL/6J mice, n = 3 per group, were orally challenged with one dose of 10^9 CFU of second generation cgt mutant, X47 HP0421::rpsL-cat-2. Mice were sacrificed two weeks after oral challenge and the bacteria load was evaluated.

5.3.3.4 Screen panel of in vitro cgt complemented constructs for in vivo complementation using the Mongolian gerbil model
Mongolian gerbil infection experiments were done in the laboratory of Professor Rainer Haas. Technical assistance was provided by Eva Loell and Florian Gilg. Outbred Mongolian gerbils (12-13 weeks of age) were orally challenged once with 10^9 CFU of H. pylori. The animals were sacrificed two weeks after challenge and the stomach was opened along the greater curvature, rinsed with sterile PBS and separated into antral and corpus tissue specimens. Tissues were homogenized (glass homogenizer, Ochs, Bovenden, Germany) in 1 ml of Brucella broth. Appropriate dilutions were spread on selective serum plates (GC agar (Oxoid, Wesel, Germany) supplemented with horse serum (8%), vancomycin (10 μg/ml), trimethoprim (5 μg/ml), nystatin (1 μg/ml), and streptomycin (250 μg/ml)), and incubated under microaerobic conditions (85% N₂, 10%
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CO₂, 5% O₂) at 37 °C for five days. Numbers of colony forming units (CFU) were expressed per gram of gastric tissue.

*H. pylori* strains used in the Mongolian gerbil experiments were passaged twice on serum plates supplemented with horse serum (8%) and streptomycin (250 μg/ml) to select for streptomycin resistance. Gerbils were challenged with wild-type X47 (naturally streptomycin resistant), a pool of Str<sup>r</sup> second generation cgt mutant strains, hAD149 and hAD150, and a pool of six different Str<sup>r</sup> uPtetO-CGT complemented second generation cgt mutant strains (Table 3-2, hAD151-hAD156). Bacteria were re-isolated on standard serum plates and serum plates supplemented additionally with chloramphenicol (10 μg/ml). Of the colonies re-isolated from gerbils challenged with pooled uPtetO-cgt complemented second generation X47 cgt mutant strains, for each infected animal, 10 colonies were selected and individually expanded. Genomic DNA was isolated from each of the 40 clones, and characterized by PCR amplification of the *mdaB*, *trpA*, *gltDH* and *HP0421* loci.

### 5.3.3.5 Colonization by tet-responsive CGT strains in vivo

Tet-responsive CGT strains were passaged on either standard CBA plates or CBA plates containing 50 ng/ml ATc for 48 h, harvested and prepared as bacterial inoculums. Starting 24 h before oral challenge, C57BL/6J mice were supplemented as indicated with ATc in a 5% sucrose solution. Animals were sacrificed two weeks after oral challenge and the bacteria load was evaluated.
5.4 Results

Several different manipulations were done to the cgt locus (HP0421) during this study. Four different cgt knockout constructs were made and a sequence encoding an HA peptide was inserted at the 3'-end of the cgt ORF in a fifth construct. All these different constructs have been compiled together in one figure for ease of reference (Figure 5-5). For clarity, the cgt gene in its native locus will be referred to as HP0421.

5.4.1 Construction of first generation cgt mutant strains

The first strategy used to generate a cgt knockout mutant in strain X47 involved making a markerless mutation by cleanly removing HP0421 from the chromosome, which in turn would permit multiple uses of the rpsL-cat cassette for subsequent manipulations within the same strain. To achieve this, two constructs were made: The first, HP0421::rpsL-cat, was made to replace a large portion of HP0421 with the rpsL-cat cassette and the second, HP0421del, was made to replace the rpsL-cat cassette and the remaining coding sequence of HP0421 to generate a final H. pylori strain X47 ΔHP0421 (Figure 5-5).

Cholesterol and its glucosylated derivatives isolated from first generation cgt mutant strains and from wild-type X47 were compared by TLC analysis. As expected, cholesteryl glucosides were absent in both the X47 HP0421::rpsL-cat-1 and X47 ΔHP0421 strains (Figure 5-6). Several X47 ΔHP0421 clones were tested for their ability to colonize C57BL/6J mice. No colonies were re-isolated one week after oral challenge.
Figure 5-5  Schematic diagram of different mutations made at the \textit{cgt} (HP0421) locus compared to the wild-type locus
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5.4.2 Complementation of first generation cgt mutant strains by insertion of uPtetO-CGT at a foreign locus

To generate *H. pylori* strains that expressed CGT in a tetracycline responsive manner, the expression of cgt was placed under the control of tet-promoters, uPtetO, (described in Chapter 4), to generate constructs uPtetO1-CGT and uPtetO2-CGT. The cgt mutant strain X47 ΔHP0421 was genetically complemented by insertion of uPtetO-CGT constructs at either the gltDH, trpA or dapB locus. This approach generated strains hAD79-hAD84 (Table 3-2) which contained one copy of cgt expressed under the control of a tet-promoter, uPtetO1 or urePtetO2. PCR positive clones were screened for the presence of cholesteryl glucosides to confirm that CGT activity was restored in these strains.

Staining with α-naphthol to detect natural cholesteryl glucosides can be problematic due to the concentration of material required. An easier method for screening *H. pylori* CGT activity was developed based on a study that demonstrated that CGT could recognize

Figure 5-6 TLC analysis of glucosylated cholesterol derivatives in first generation *H. pylori* cgt mutants

*H. pylori* X47 wild-type and first generation cgt mutants, X47 HP0421:: rpsL-cat-1 and X47 ΔHP0421, were grown up in HI media for 24 h. Lipids were extracted, separated by TLC in chloroform/methanol 85:15 (v/v) and visualized by staining with α-naphthol.

- Lane 1: αCG synthetic standard
- Lane 2: αCAG synthetic standard
- Lane 3: *H. pylori* X47 wild-type strain
- Lane 4: *H. pylori* X47 HP0421::rpsL-cat-1
- Lane 5: *H. pylori* X47 ΔHP0421
and glucosylate a fluorescently labelled cholesterol derivative, 25-NBD-cholesterol (Wunder et al., 2006). The NBD (nitrobenzoxadiazole) unit of 25-NBD-cholesterol is a fluorophore and is linked to C25-atom of the fatty acid chain. This compound could be loaded into the bacterial membrane and extracted after several hours of incubation. Lipid extracts containing NBD-cholesterol and its derivatives could then be separated by TLC and visualized by exciting the fluorophore and detecting its emission. Based on this precedent, the systematic use of NBD-cholesterol as a reporter for routine detection of CGT activity was investigated. To confirm that NBD-cholesterol could act as substrate for CGT, wild-type and CGT knockout strains were grown in media supplemented with or without NBD-cholesterol. Lipid extracts were separated by TLC and the plate was first visualized by light excitation and later stained with α-naphthol (Figure 5-7 A and B). Side by side comparison of the images demonstrated that NBD-cholesterol was converted to the equivalent major H. pylori cholesteryl glucoside derivatives and that the NBD-cholesteryl glucosides co-migrated with similar RfS as their unlabelled cholesterol counterparts, consistent with that found in the literature (Wunder et al., 2006). Lipid extracts from the cgt mutant strain incubated with NBD-cholesterol only contained the unmodified substrate. Glycosylation of NBD-cholesterol by CGT resulted in the formation of NBD-αCG, which could be detected within 4 h of incubation with wild-type H. pylori, while the NBD-αCAG derivative was detected after 24 h of incubation (Figure 5-8 A). Labelling with NBD-cholesterol proved to be a more efficient method for detecting CGT activity and therefore was used to screen uPtetO-CGT strains for enzymatic complementation in vitro (Figure 5-8 B). Positive clones were identified for all complemented strains (Table 3-2, hAD79-hAD84).

Before these strains could be converted into conditional cgt mutants, by the introduction of the tet-repressor (ptetR, section 4.4.1), they were evaluated for complementation in vivo by testing if their ability to colonize mice had been restored. Surprisingly, none of the complemented cgt mutants could be re-isolated from mice stomachs two weeks after oral challenge. Since CGT activity had been restored in these strains, their lack of colonization suggested that manipulation of the cgt locus may have resulted in polar effects on neighbouring genes, or that complementation with the uPtetO-CGT constructs may have caused an imbalance of cholesteryl glucosides that was detrimental to the bacteria in vivo.
Figure 5-7 Validation for the use of NBD-cholesterol to detect CGT activity

TLC analysis of lipids extracted from *H. pylori* X47 wild-type and *cgt* mutant strain grown with or without NBD-cholesterol.

- Lane 1: NBD-Ch standard
- Lane 2: *H. pylori* X47 ΔHP0421 + NBD-Ch
- Lane 3: *H. pylori* X47 ΔHP0421
- Lane 4: *H. pylori* X47 wild-type strain + NBD-Ch
- Lane 5: *H. pylori* X47 wild-type strain
- Lane 6: αCAG synthetic standard
- Lane 7: αCG synthetic standard

The TLC plate was first (A) visualized for fluorescence and then (B) stained with α-naphthol. Both natural and NBD labelled cholesteryl glucosides are absent in the lipid extracts of strain X47 ΔHP0421. NBD-Ch: NBD-cholesterol, NBD-αCG: NBD-cholesteryl α-D-glucopyranoside, NBD-αCAG: NBD-cholesteryl 6-O-tetradecanoyl-α-D-glucopyranoside, NBD-αCPG: NBD-cholesteryl 6-O-phosphatidyl-α-D-glucopyranoside.
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**Figure 5-8 Assay for CGT activity using NBD-cholesterol**

A. NBD-cholesterol is taken up by *H. pylori* and converted to glucosylated derivatives over time. Wild-type X47 was grown in Brucella broth to mid-log phase before cultures were inoculated with 1 μg/ml NBD-cholesterol. Cultures were incubated for a further 0, 4, 8, 12, and 24 h. Fluorescent spots NBD-αCG and NBD-αCAG that migrate below the NBD-Ch standard (M) appear at 4 and 24 h after incubation with *H. pylori*.

B. TLC analysis of lipid extracts of *H. pylori* X47 wild-type (WT) and *uPtetO-CGT* complemented X47 ΔHP0421 strains grown in Brucella broth with NBD-cholesterol.

Lane M: NBD-Ch standard
Lane WT: *H. pylori* X47 wild-type strain
Lane 1: *H. pylori* X47 ΔHP0421; trpA::uPtetO1-CGT (hAD79)  
Lane 2: *H. pylori* X47 ΔHP0421; trpA::uPtetO2-CGT (hAD80)  
Lane 3: *H. pylori* X47 ΔHP0421; gltDH::uPtetO1-CGT (hAD81)  
Lane 4: *H. pylori* X47 ΔHP0421; gltDH::uPtetO2-CGT (hAD82)  
Lane 5: *H. pylori* X47 ΔHP0421; dapB::uPtetO1-CGT (hAD83)  
Lane 6: *H. pylori* X47 ΔHP0421; dapB::uPtetO2-CGT (hAD84)
5.4.3 Construction of second generation X47 cgt mutant strains

The first attempt at making an X47 cgt mutant and complementing it with a uPtetO-CGT construct at a foreign locus in the chromosome restored CGT activity but failed to restore colonization. A cgt mutant in H. pylori strain Hp76 has previously been successfully complemented both in vitro and in vivo (Wunder et al., 2006). This mutant was generated by replacing HP0421 with a chloramphenicol resistance cassette, and then complemented by expressing cgt on the pHel3 plasmid. In the case of the Hp76 cgt mutant, it may be that the resistance cassette used to replace HP0421 could maintain good expression of the downstream HP0422 gene, while for the first generation cgt mutant constructed in X47, the complete removal of HP0421 may have damaged the HP0417-HP0422 operon, reducing expression of HP0422 and thus preventing colonization.

Therefore, the new constructs for making second generation cgt mutant strains were designed to promote expression of downstream HP0422 ORF by the strong cat promoter in the rpsL-cat cassette. To facilitate translation of HP0422, a BamHI restriction site (GGATCC) was added immediately after the last three base-pairs of the rpsL-cat cassette (TAA) to create a consensus H. pylori Shine-Dalgarno sequence (TAAGGATCC) (Sharma et al., 2010). The constructs were designed so that insertion of BamHI flanked rpsL-cat cassette formed a Shine-Dalgarno sequence at an appropriate distance from a downstream ORF (e.g., AAGGAT-(6/7nn)-ATGNNNN). For construct CGT-Δ2-RCAT, the inserted BamHI flanked rpsL-cat cassette generated a Shine-Dalgarno sequence 7 nucleotides before the last methionine encoding codon of the HP0421 ORF, resulting in an ORF encoding the last 43 aa of CGT. For construct CGT-Δ3-RCAT, the BamHI flanked rpsL-cat cassette was inserted to preserve the Shine-Dalgarno sequence located 6 bp upstream of the HP0422 ORF. Transformation of wild-type X47 with these redesigned constructs generated second generation cgt mutant strains X47 HP0421::rpsL-cat-2 and X47 HP0421:: rpsL-cat-3 (Figure 5-5).

Cholesterol and its glucosylated derivatives were isolated from strains X47 HP0421::rpsL-cat-2, X47 HP0421:: rpsL-cat-3 and wild-type X47, and compared by TLC analysis. As expected glucosylated cholesterol derivatives were absent in both cgt mutant strains, consistent with that observed for the first generation X47 cgt mutants. These strains were also tested for their ability to colonize C57BL/6J mice and outbred
Mongolian gerbils. For both animal models, no *H. pylori* colonies could be re-isolated two weeks after oral challenge with second generation X47 cgt mutants.

### 5.4.4 Complementation of second generation cgt mutant strains

#### 5.4.4.1 Complementation of second generation cgt mutants with *uPtetO*-*CGT* in vitro

Since the counterselection cassette remained in the second generation cgt mutant strains, additional DNA manipulations using the *rpsL-cat* cassette could not be done in these strains. Complementing these strains by introducing a copy of cgt on a plasmid was not feasible, as strain X47 does not easily take up plasmids (Ondek Pty. Ltd., unpublished data). A second counterselectable marker for genetic manipulation of *H. pylori* was not available and consequently any additional genetic manipulations of second generation cgt mutant strains using the *rpsL-cat* replacement strategy had to be done before the mutation in the cgt locus was made. Therefore, to generate genetically complemented second generation cgt mutants, a second copy of cgt, in the form of *uPtetO*-CGT, was first inserted at either the *gltDH* or *trpA* locus followed by replacement of the native HP0421 gene, in the cgt locus by *rpsL-cat* using either pCGT-Δ2-RCAT or pCGT-Δ3-RCAT. Though this was not the optimal strategy, great care was taken to ensure that the desired mutations were made and that they were stable. The final Cm r strains were characterized by PCR to ensure that *uPtetO*-CGT was still at the recipient locus and that *rpsL-cat* had replaced the native HP0421 ORF (sections 3.3.3.2 and 3.3.4.1). Strains were also screened for CGT activity as had been done for the *uPtetO*-CGT complemented first generation X47 cgt mutants.

#### 5.4.4.2 Screen of *uPtetO*-CGT complemented X47 HP0421::*rpsL-cat*(2/3) strains for complementation in vivo using the Mongolian gerbil infection model

As no published data was available on the wild-type levels of endogenous CGT expression and the number of gerbils was limited, three *uPtetO*-CGT-locus combinations were selected for screening cgt complementation in vivo. The combinations were selected to cover the broad range of *uPtetO* driven expression that could be achieved with the *tet*-system developed for *H. pylori* (Chapter 4). The constructs *uPtetO1*-CGT and *uPtetO2*-CGT inserted at the *trpA* locus represented the highest and lowest levels of CGT expression respectively, and the *uPtetO1*-CGT
construct inserted at the gltDH locus represented an intermediate level of CGT expression. Furthermore, it was also unknown which of the two second generations cgt mutants, if either, could indeed be complemented in vivo. Therefore a pool of six complemented cgt mutants was used to simultaneously test which cgt mutant (HP0421::rpsL-cat-2 or HP0421::rpsL-cat-3) could be complemented and which uPtetO-CGT–locus combination (trpA::uPtetO1-CGT, trpA::uPtetO2-CGT or gltDH::uPtetO1-CGT) could efficiently complement the cgt mutation in vivo. To easily differentiate between the uPtetO1-CGT construct and uPtetO2-CGT construct inserted at the trpA locus in any colonies re-isolated from gerbil stomachs, strains harbouring the uPtetO2-CGT construct also harboured the ptetR1 construct (expressing revTetR) at the mdaB locus while strains harbouring the uPtetO1-CGT construct were genotypically wild-type at this locus (Table 5-1).

![Table 5-1](image)

The H. pylori strains that were to be tested in the Mongolian gerbil experiments (Table 3-2, hAD110-hAD113, hAD117, hAD118, hAD124 and hAD125) all harboured the rpsL-cat cassette and were therefore sensitive to streptomycin. However the isolation protocol in the Haas laboratory required selective serum plates which contained streptomycin. Therefore these eight strains were passaged twice on serum plates supplemented with horse serum (8%) and streptomycin (250 μg/ml) to select for streptomycin resistance by conversion of the wild-type rpsL allele on the rpsL-cat cassette to the mutant allele harboured in the X47 chromosome (Table 3-2, hAD149-hAD156). Gerbils were orally challenged with wild-type X47, a pool of Str² second generation cgt mutant strains, (hAD149 and hAD150), or a pool of six different Str² uPtetO-CGT complemented second generation cgt mutant strains (Table 5-1, hAD151-hAD156).
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The infection status and colonization load in both the corpus and the antrum were evaluated two weeks after challenge. Colonies were re-isolated from all four animals challenged with the pool of complemented *cgt* mutants. Consistent with published data (Rieder et al., 2007), the bacterial load was greater in the corpus than in the antrum (Figure 5-9). In antrum tissue, the bacterial load in animals infected with the complemented strains was approximately one log lower than that of animals infected with wild-type X47, however in the corpus tissue the bacterial load was similar. PCR fingerprinting of 10 colonies re-isolated from each infected gerbil revealed that the animals were infected with a mixture of two to four strains (Figure 5-10 and Table 5-2). At least five of the six complemented *cgt* mutant strains tested were successfully re-isolated. The identity of 1 out of the 40 fingerprinted colonies could not be confirmed due to a failed PCR reaction; however its identity could be narrowed down to either strain hAD151 or strain hAD155.

![Figure 5-9](image)

**Figure 5-9** Colonization of *uPtetO-CGT* complemented X47 *cgt* mutants in Mongolian gerbils

Three groups of Mongolian gerbils were infected with wild-type X47 (WT), a pool of second generation *cgt* mutants (ΔCGT) or a pool of 6 different strains (CGT comp) that expressed *cgt* from a foreign locus under the control of the *uPtetO* promoter. Colonization was evaluated two weeks after oral challenge. The bacterial load in gerbils infected with CGT comp strains was similar to that of gerbils infected with wild-type X47 strain, in both the antrum and the corpus. *H. pylori* colonies could not be re-isolated from gerbils challenged with X47 *cgt* mutants. Horizontal bars represent mean bacterial load per group (n = 3 or 4) and points plotted represent colonization density for each individual animal. Detection limit was < 100 CFU/g of stomach tissue (dotted horizontal line). Gastric specimens without *H. pylori* re-isolation are shown as null.
Four Mongolian gerbils were each infected with a pool of 6 different strains that express cgt from a foreign locus under the control of a uPtetO promoter. All four gerbils were successfully colonized two weeks after oral challenge. Ten clones (1-10) were picked from each isolation plate and grown up for genomic DNA characterization. PCR amplification of four different loci provided the information required to differentiate between the 6 different strains tested. The PCR products amplified for each locus for each colony are presented and compared to wild-type X47 strain (C).
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Table 5-2  Identity of strains re-isolated from infected Mongolian gerbils

<table>
<thead>
<tr>
<th>Gerbil 1</th>
<th>Gerbil 2</th>
<th>Gerbil 3</th>
<th>Gerbil 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain identity</td>
<td>Fraction</td>
<td>Strain identity</td>
<td>Fraction</td>
</tr>
<tr>
<td>4</td>
<td>3/10</td>
<td>2</td>
<td>3/10</td>
</tr>
<tr>
<td>5</td>
<td>5/10</td>
<td>4</td>
<td>3/10</td>
</tr>
<tr>
<td>6</td>
<td>1/10</td>
<td>6</td>
<td>4/10</td>
</tr>
<tr>
<td>1 or 5</td>
<td>1/10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These results demonstrated that, unlike the first generation *cgt* mutant constructs, the second generation *cgt* mutant constructs did not negatively affect the surrounding genes in the *HP0421* locus. Furthermore, both *uPtetO-CGT* constructs could drive sufficient expression of CGT to complement the *cgt* mutants *in vivo*. Fingerprint PCR analysis identified 90% of the characterized colonies as strains that express CGT in the low to medium range of possible expression levels, while only 7.5% of the re-isolated colonies were identified as strains that expressed high levels of CGT. Overall these results demonstrated that the second generation *cgt* knockout strains could be complemented by inserting *uPtetO-CGT* constructs at a foreign locus and that the strategy for regulating *cgt* expression had potential.

5.4.5 Quantifying wild-type levels of CGT expression

Little is known about CGT’s expression or its catalytic efficiency *in vitro*. Thus, in addition to validating the *tet*-system as a genetic tool to regulate CGT expression, the different *tet*-promoters were also used to correlate CGT protein levels with the relative amounts of cholesteryl glucosides. To achieve this goal, a variant of the *uPtetO-CGT* construct, *uPtetO-CGT-HA*, was made which contained an additional 33 bp of DNA after the HP0421 ORF. This construct expressed CGT-HA, a full length CGT protein with a C-terminal HA peptide tag (SFERFEIFPKE). The HA peptide is derived from the influenza hemagglutinin (HA) molecule H1(aa 110-119) from the PR8 strain and is recognized by the antibody produced by the CM1-1 hybridoma cell line. In addition, to put *tet*-regulated CGT expression in context with wild-type levels of CGT expression, it was necessary to make an X47 strain which expressed the HA-tagged enzyme from its native locus (Figure 5-5).
Strain X47 *HP0421::HA* was made for this purpose. Expression of CGT-HA in X47 *HP0421::HA* transformants was successfully detected by immunoblotting (Figure 5-11 A). The presence of the C-terminal HA peptide did not impede the activity of the enzyme as there was no significant difference in the amount of cholesteryl glucoside derivatives in the total lipids extracted from X47 *HP0421::HA* compared to wild-type X47 grown under the same conditions (Figure 5-11 B). This demonstrated that strain X47 *HP0421::HA* could be used to compare the amount of CGT-HA (and hence CGT) produced by wild-type and *uPtetO-CGT-HA* (equivalent to *uPtetO-CGT*) complemented strains.

### 5.4.6 Generation of conditional CGT strains

To validate the *H. pylori* tet-system developed in Chapter 4 as a potentially useful genetic tool for regulating CGT expression, *tet*-regulated CGT expression was characterized by monitoring changes in CGT protein expression and by analyzing production of the enzyme product, αCG.

#### 5.4.6.1 Generation of *tet*-responsive *cgt* strains

As discussed in section 5.4.4.1, in order to use the second generation *cgt* mutant constructs to inactivate *HP0421* in its native locus, any additional manipulations at other chromosomal loci had to be done beforehand. Therefore recipient strains expressing TetR, (Table 3-2 hAD17–29) were first transformed with the appropriate *uPtetO-CGT* or *uPtetO-CGT-HA* plasmids to generate strains that express both TetR and two copies of *cgt* (Table 3-2, hAD99-109 and Table 3-5, hAD157-169). These strains were then transformed using pCGT-Δ2 or pCGT-Δ3 to replace *HP0421* at its native locus with *rpsL-cat* to obtain strains that expressed either *cgt* or *cgt-HA* from a foreign locus in a tetracycline dependent manner (Table 3-2, hAD122-148, and Table 3-5, hAD170-175).
Figure 5-11 Tagged native HP0421 is functionally active

Wild-type X47 (WT), X47 HP0421::rpsL-cat-3 mutant (Δ3) and X47 HP0421::HA clones expressing CGT tagged with a C-terminal HA peptide from the wild-type locus (1-3) were grown in HI media for 24 h. The cultures were divided equally and bacteria were washed and pelleted. (A) One bacterial pellet was used for immunodetection of HA tagged CGT. A band corresponding to the predicted MW of 45 kDa was evident in lanes with X47 HP0421::HA cell lysate. (B) Lipids were extracted from the second bacterial pellet and analysed by TLC using α-naphthol stain. No significant difference in the amounts of cholesteryl glucosides was observed between wild-type X47 and X47 HP0421::HA strains. Synthesized standards: αCG and αCAG (M).
5.4.6.2 In vitro characterization of conditional cgt strains

Tet-regulation of CGT expression in uPtetO-CGT complemented cgt mutants expressing TetR (Table 3-2 hAD122-148, Table 3-5, hAD162-175) was characterized by analysing changes in both protein expression and by assaying for changes in enzyme activity by comparing lipid extracts of conditional CGT expressing strains grown in HI media with or without 100 ng/ml of ATc.

TLC analysis of lipid extracts from strains transformed with uPtetO1-CGT or uPtetO2-CGT constructs showed that cholesterol derivatives were still being produced in the absences of ATc (Figure 5-12 A). This meant one of two possible explanations, that CGT expression from the uPtetO-CGT constructs was not being regulated by TetR and therefore was constitutively expressed or, alternatively, CGT expression was down regulated but the residual amount of CGT being expressed was sufficient to synthesize the amount of αCG required by the bacterium under the growing conditions tested. Immunodetection of CGT-HA expression from uPtetO1 and uPtetO2 tet-promoters inserted at the trpA locus demonstrated that the latter situation was the case (Figure 5-12 B and C). CGT-HA induction was time dependent and complete 24 h after induction with ATc. As observed earlier in the GFP studies (Chapter 4), in tet-regulated strains harbouring uPtetO1-CGT-HA, CGT-HA protein levels could not be induced to the same protein levels as detected in strains lacking TetR, and induction in ptetR4 strains was better than in ptetR2 strains. Despite the evidence of tet-regulation, low levels of CGT-HA could still be detected when these strains were grown without ATc.

In an attempt to make strains that repressed CGT expression more stringently, a tet-promoter containing three tetO sites was tested (uPtetO3, Chapter 4). The uPtetO3-CGT-HA construct was inserted at both the trpA and the weaker gltDH locus in strains expressing TetRs (Table 3-5, hAD164, hAD165, hAD168, hAD169). Despite the addition of a third tetO binding site, induction of uPtetO3 was still possible (Figure 5-13 A and B). Repression of CGT-HA expression was most efficient in strain hAD173, expressing TetR under the amiE promoter (ptetR2) and CGT-HA under uPtetO3 at the gltDH locus. However when compared directly to the ‘wild-type’ amount of CGT-HA protein expressed in strain X47 HP0421::HA, the residual amount of CGT-HA expressed by these tet-responsive strains in the repressed state was just below or equal to that of strain X47 HP0421::HA. A final comparison of CGT-HA expression was done between ‘wild-type’ and the repressed and induced states of all
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*uPtetO*(1-3)-CGT-HA strains transformed with the same *ptetR* construct (Figure 5-14). The data demonstrated that, in all strains tested, induction of *uPtetO*(1-3) by ATc resulted in over expression of CGT-HA, while in the repressed state the amount of CGT-HA protein in *ptetR*2; *uPtetO-CGT-HA* strains was comparable to ‘wild-type’ levels of CGT-HA expression. Furthermore, addition of a third tetO binding site in *uPtetO*3 did not significantly alter the properties of the promoter as CGT-HA levels were very similar for strains with either *uPtetO*2 or *uPtetO*3, in both the repressed and induced states. Under the conditions tested, CGT-HA expression by tet-responsive *uPtetO*(1-3) promoters could not be reduced below ‘wild-type’ levels of expression.

In light of the evidence that CGT expression is significantly increased in *uPtetO*1-CGT strains upon induction with ATc (Figure 5-14), careful analysis of the TLC data suggests that this large increase in CGT expression may have altered the relative proportions of each cholesterol derivative in the bacterial membrane as *uPtetO*1-CGT strains, hAD128 and hAD133, had less αCAG and more of αCG when grown with ATc than when grown without ATc (Figure 5-12 A). This change in the relative proportions of each cholesterol derivative upon ATc induction was not observed in strains hAD130, hAD173-hAD175 which had either *uPtetO*2 or *uPtetO*3 driving CGT expression (Figure 5-12 A and Figure 5-13 C and D).

Though CGT expression levels could not be reduced below wild-type levels under *in vitro* growth conditions, whether this was the case *in vivo* remained to be tested, as the relative amounts of each cholesteryl glucoside in the *H. pylori* membrane can vary depending on the growth environment. TLC analysis of cholesteryl glucosides revealed that bacteria grown in Brucella broth had more of the αCG derivative than αCAG compared to bacteria grown in BHI or HI (Figure 5-15). This difference may be attributed to the presence of bisulfite in the Brucella broth which has been demonstrated to inhibit *H. pylori* growth (Hawrylik et al., 1994). However, since *in vivo* conditions are quite different to *in vitro* culture and transcription of both the HP0417-HP0422 operon and HP0420-HP0422 suboperon increase under acidic stress (Sharma et al., 2010), it was worth investigating if regulation of CGT expressing *in vivo* had an effect on colonization.
Figure 5-12 Tet-responsive induction of uPtetO1-CGT and uPtetO2-CGT
Figure 5-12 *Tet*-responsive induction of *uPtetO1-CGT* and *uPtetO2-CGT* (adjacent page)

(A) TLC analysis of lipids extracted from wild-type X47 (WT), X47 HP0421::rpsL-cat-3 mutant (Δ3) and conditional *cgt* mutant strains hAD128, hAD130 and hAD133 (Table 3-2). Bacteria were grown in HI media to mid-log phase (OD600 = 0.5) before 100 ng/ml ATc was added (+) and incubated for an additional 24 h. Induction with ATc resulted in increased amounts of αCG in strains expressing *uPtetO1-CGT* (hAD128 and hAD133), however in all complemented strains cholesteryl glucosides were still synthesized in the repressed state (-). Synthesized αCG standard (M).

(B and C) Detection of HA tagged CGT by immunoblot assay. Bacteria were transformed as indicated and grown in HI media to mid-log phase (OD600 = 0.5) before 100 ng/ml ATc was added. Aliquots of induced cultures were taken at indicated time points. Lysates from *H. pylori* expressing CGT-HA from *tet*-responsive promoters, *uPtetO1* and *uPtetO2*, were separated on a 10% SDS–PAGE gel. Equal amount of protein was loaded into each lane. CGT-HA is a 45 kDa protein, indicated by the black arrows.

(B) Induction of CGT-HA in strains with *P_amIE* driving expression of TetR (*ptetR2*). Lane 1, wild-type X47 (neg); Lane 2, constitutively expressed CGT by X47 strain lacking TetR (pos); Lanes 3 and 6, repressed CGT-HA (+ TetR); lanes 4 and 7, 12 h after induction with ATc; lanes 5 and 8, 24 h after induction.

(C) Similar to (B), Induction of CGT-HA in strains with *P_flA* driving expression of TetR (*ptetR4*).
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**Figure 5-13**  
*Fig. 5-13* Tet-induction of uPtetO3-CGT-HA
Figure 5-13  *Tet*-induction of *uPtetO3-CGT-HA* (adjacent page)

(A) Immunodetection of HA-tagged CGT expressed by conditional *cgt* mutant strains complemented with *uPtetO3-CGT-HA* at the *trpA* locus or (B) at the *gltDH* locus. Bacteria were transformed as indicated and grown in HI media to mid-log phase (OD$_{600} = 0.5$) before 100 ng/ml ATc was added (+). Aliquots of induced cultures were taken at indicated time points. Lysates from *H. pylori* expressing CGT-HA from *tet*-responsive *uPtetO3* promoter were separated on a 10% SDS–PAGE gel. Equal amount of protein was loaded into each lane. Lane 1 and 4, repressed CGT-HA (+ TetR, - ATc); Lanes 2 and 5, 12 h after induction; Lanes 3 and 6, 24 h after induction. Lane 7, constitutively expressed CGT-HA by X47 lacking TetR (pos); Lane 8, wild-type X47 (neg); Lane 9, strain X47 *HP0421::HA* expressing CGT-HA from the native locus (ntv). CGT-HA is a 45 kDa protein, indicated by the black arrows.

(C and D) TLC analysis of lipids extracted from wild-type X47 (WT), X47 *HP0421::rpsL-cat-3* mutant (Δ3) and conditional *cgt* mutants complemented with *uPtetO3-CGT-HA*. Bacteria were grown in HI media to mid-log phase (OD$_{600} = 0.5$) before 100 ng/ml ATc was added (+) and incubated for an additional 24 h. No significant changes in cholesteryl glucosides were observed upon induction with ATc. Synthesized αCG and αCAG standards (M).
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Figure 5-14 Comparison of CGT regulation by different *tet*-responsive *uPtetO*-CGT strains

Bacteria were transformed as indicated and grown in HI media to mid-log phase (OD$_{600}$ = 0.5) before 100 ng/ml ATc was added and incubated for an additional 24 h. Lysates from *H. pylori* expressing CGT-HA from *tet*-responsive *uPtetO* promoters were separated on a 10% SDS–PAGE gel. Equal amount of protein was loaded into each lane. CGT-HA is a 45 kDa protein, indicated by the black arrows.

(A) Induction of CGT-HA by strains with *P$_{amiE}$* driving expression of TetR (*ptetR2*). Specific conditions

- Lanes 1 and 2: *uPtetO1*-CGT inserted at the *trpA* locus
  - Lane 1: repressed (-), Lane 2: induced with 100 ng/ml ATc (+)
- Lanes 3 and 4: *uPtetO2*-CGT inserted at the *trpA* locus
- Lanes 5 and 6: *uPtetO3*-CGT inserted at the *trpA* locus
- Lanes 7 and 8: *uPtetO3*-CGT inserted at the *gltDH* locus
- Lane 9: X47 HP0421::HA (standard for wild-type levels of cgt expression) (ntv)

(B) Induction of CGT-HA by strains with *P$_{fluA}$* driving expression of TetR (*ptetR4*).
Figure 5-15  Cholesteryl glucoside derivatives in different media
TLC analysis of lipids extracted from wild-type X47 (WT) and X47 HP0421::rpsL-cat-3 mutant (Δ3) grown in BHI, Brucella broth or HI media with 1 μg/ml of NBD-cholesterol for 24 h. Bacteria were harvested and lipids were extracted and separated by TLC. (A) TLC was stained with α-naphthol to visualize total cholesteryl glucosides. (B) NBD-cholesterol derivatives were visualized under light. Different growth media affect the relative amounts of different cholesteryl glucosides in the membrane of H. pylori.

5.4.6.3 Colonization of conditional CGT strains
Despite the inability of the tet-system to efficiently repress CGT expression in vitro, it was still a useful tool to test if preventing H. pylori from adjusting CGT expression levels during infection had an adverse effect on colonization. The most stringent and weakest CGT expressing strain as determined by immunoblot data, hAD173, was grown on standard CBA plates or induced for 48 h on plates containing 50 ng/ml ATc. Mice supplemented with either 0 or 1 μg/ml ATc in their drinking water were orally challenged with these strains and the colonization status and bacterial load was evaluated two weeks later. All animal groups were found to be colonized; however, the bacterial load was significantly lower in mice infected with bacteria that were induced prior to oral challenge compared to the animal groups infected with bacteria that were not induced before challenge (i.e., had wild-type amounts of CGT) (Figure 5-16).
Figure 5-16 Colonization of C57BL/6J mice by conditional CGT expressing strains

The most stringent conditional cgt expressing strain as identified by immunoblot characterization, X47 mdaB::ptetR2; gltDH::uPtetO3-CGT-HA; HP0421::rpsL-cat3 (hAD173, Table 3-5) was used to evaluate if cgt could be sufficiently repressed to prevent colonization. Mice were separated into three groups of n = 5. Two groups of mice were challenged with untreated bacteria and had either no supplement in their water (-/-), or were supplemented with ATc (-/+). The third group was challenged with bacteria induced with 50 ng/ml ATc for 48 h prior to oral challenge and were supplemented with ATc in their drinking water (+/+). H. pylori colonies were re-isolated from all three groups of mice. Horizontal bars represent mean bacterial load per group, error bars represent standard deviation and points plotted represent colonization density for each individual animal. Detection limit was < 20 CFU per stomach (dotted horizontal line). Gastric specimens without H. pylori re-isolation are shown as null. Statistical significance was evaluated using a Student’s t-Test ** P < 0.01, *** P < 0.0001.
5.5 Discussion

The aim of this study was to generate a conditional *H. pylori* cgt mutant using the tet-system develop for *H. pylori* (Chapter 3) in order to study the role of CGT and cholesteryl glucosides in persistence and immune modulation *in vivo*. Several challenges were encountered during this study, some of which were successfully overcome, however a conditional cgt mutant was not achieved using the tet-responsive *uPtetO* promoters. Nevertheless, interesting insights were gained into cgt expression and the biological importance of cholesteryl glucosides. The study also gave insight into future directions for the improvement of the *uPtetO* promoters and for more stringent repression.

The first challenge encountered during this study was the construction of an X47 cgt mutant that could be complemented. Complete removal of the HP0421 ORF along with the non-coding 4 bp sequence between the HP0420 and HP0421 ORFs resulted in a first generation cgt mutant that lacked cholesteryl glucosides and which could not be re-isolated from mouse stomachs 2 weeks after challenge. Transformation of this cgt mutant with the *uPtetO-CGT* constructs led to restoration of cholesteryl glucoside biosynthesis but not colonisation. Since CGT activity had been restored in these strains, the incomplete complementation indicated that manipulation of the cgt locus may have resulted in polar effects on neighbouring genes or that complementation with the *uPtetO-CGT* constructs may have caused an imbalance in the regulation of cholesteryl glucosides that was detrimental to the bacteria *in vivo*. The *uPtetO-CGT* constructs successfully complemented the second generation cgt mutants both *in vitro* and *in vivo*, indicating that complementation of the first generation cgt mutants was most likely incomplete due to the disruption or loss of important sequences originally in the HP0421 ORF, which resulted in polar effects. CGT mutants previously generated in strains Hp27 and G27 were made by replacing the HP0421 ORF with a chloramphenicol resistance cassette (*cat*) (Hildebrandt and McGee, 2009; Wunder et al., 2006), and the Hp27 cgt mutant was successfully complemented by expressing cgt on the pHel3 plasmid. In the case of the Hp27 cgt mutant, it can be hypothesised, based on the limited information provided about its construction, that complete complementation of the mutant was possible because the resistance cassette could maintain good expression of the downstream HP0422 gene. While in the case of the first generation X47 cgt mutant generated in this study, X47 ΔHP0421, the genetic manipulation at the
cgt locus may have damaged the HP0417-HP0422 operon, reducing expression of HP0422 and thereby affecting colonization. Therefore, to mimic the successful strategy used in Hp27, second generation cgt mutant constructs were designed to utilize the strong cat promoter in rpsL-cat to drive expression of HP0422 upon replacement of cgt with the counterselection cassette. Both second generation cgt mutant constructs generated H. pylori X47 cgt mutant strains in which both cholesteryl glucosides biosynthesis and colonization could be restored by complementation with the uPtetO-CGT constructs.

The next challenge in this study involved generating conditional cgt mutants that, upon induction with ATc/Dox, expressed wild-type levels of CGT. Complementation of the Hp76 cgt mutant was achieved by expressing cgt from the strong flaA promoter on the pHel3 plasmid, however the TLC data in that study suggested that the strain may have overexpressed CGT (Wunder et al., 2006). X47 strains that express gfpmut2 from the flaA promoter on the pHel2 plasmid are more fluorescent than strain X47 trpA::uPtetO1-GFP (hAD11, Chapter 4) (Dr. Phebe Verbrugghe, private communication), and therefore expression of cgt from uPtetO-CGT inserted in the chromosome was likely to be much lower than from the pHel3 plasmid construct. To attain wild-type levels of CGT complementation using uPtetO-CGT, different uPtetO-locus combinations were selected to cover the range of expression levels that could be achieved with the tet-system developed in Chapter 4.

Unfortunately, even with the addition of a third tetO binding site in uPtetO3 promoter, TLC analysis demonstrated that X47 strains, expressing both TetR and uPtetO regulated cgt, were still able to biosynthesize cholesteryl glucosides in the repressed state. This raised questions about whether CGT expression was in fact being regulated at all, and about what the CGT protein levels were in comparison to wild-type X47. Antibodies against CGT were not readily available, but terminal peptide tags were shown not to interfere with CGT activity (Lebrun et al., 2006), and therefore CGT was tagged with a C-terminal HA peptide in both wild-type and conditional cgt strains to investigate. Immunoblot data of CGT-HA expression demonstrated that cgt expression could indeed be regulated in a tetracycline dependent manner, however residual transcription form silenced uPtetO promoters resulted in CGT protein levels that were equivalent to wild-type expression levels. To date, little data has been published regarding the endogenous levels of CGT protein in H. pylori. Data from the study conducted here suggests that
CGT protein levels are quite low within the bacterium which is surprising as up to 25% of the entire cell membrane is composed of cholesteryl glucosides. Furthermore, as only the membrane form (constituting ~ 50% of total CGT protein) is active (Hoshino et al., 2011), this seems to suggest that the enzyme is very efficient and therefore it would be interesting to study CGT’s catalytic properties in more detail.

Consequently, due to the relatively high levels of residual cgt transcription, induction of uPtetO resulted in over expression of CGT protein. Increased CGT expression did not translate to significantly higher amounts of cholesteryl glucosides, as a majority of cholesterol is usually in the glucosylated form (Haque et al., 1996; Haque et al., 1995). However, over expression of CGT may have changed the relative proportions of each cholesteryl glucoside derivative in the bacterial membrane. The morphological transition from the spiral to the coccoid from of H. pylori is associated with an increase in the proportion of αCPG and a decrease in the proportion of αCG in the bacterial membrane (Shimomura et al., 2004). The coccoid forms of H. pylori are thought to contribute to modulation of the host immune response and to be important for persistence (Bonis et al., 2010; Chaput et al., 2006). A recent study demonstrated that intestinal DCs in the Peyer’s patches capture the coccoid form of H. pylori and direct H. pylori-specific T cell priming (Nagai et al., 2007). Apart from the involvement of AmiA, the molecular mechanisms for the morphological transition between the spiral and the coccoid forms still remain largely unknown (Chaput et al., 2006). The over expression of CGT may result in the inappropriate biosynthesis of cholesteryl glucoside derivatives, which may disrupt the regulated transition between the spiral and coccoid forms, and consequently inhibit immune modulation and persistence mechanisms. CGT over expression did not result in any noticeable phenotype in vitro; however, data from the competition experiment between strains expressing different levels of CGT (section 5.4.4.2), and the infection experiment using tet-regulated cgt strains in mice (Figure 5-16) suggest that high levels of CGT expression are unfavourable for H. pylori to establish an infection. Although only 40 of the colonies re-isolated from gerbils were completely characterized, 90% were identified as strains with low-medium levels of CGT expression and both strains expressing high levels of CGT, hAD151 (1) and hAD152 (2), were under-represented among the re-isolates. Furthermore, in the mouse model, induction of CGT expression prior to oral challenge resulted in a lower bacterial burden compared to the animal groups challenged with strains that were not induced.
A very recent publication by McGee et al. and data from our laboratory also suggests that cholesteryl glucosides have a role in establishing infection. Previously published work (Wunder et al., 2006) and the experiments in this study identified the *H. pylori cgt* mutant as a non-colonizer when colonization status was evaluated within 2 weeks of oral challenge. Yet, when the colonization was evaluated at 4 weeks after infection, the *H. pylori SS1 cgt* mutant could be re-isolated from Mongolian gerbils (McGee et al., 2011), and strain X47 Δ*HP0421* could be re-isolated from C57BL/6J mice (Ondek Pty. Ltd., unpublished data). However, in both animal models, the bacterial load of the *cgt* mutants was significantly impaired when compared to the wild-type strain. These new data suggest that the *cgt* mutant requires significantly more time to establish an infection. It would be interesting to test if the *cgt* mutant is permanently impaired, or if given enough time, it can eventually establish a robust infection and attain a wild-type bacterial load. If the mutant remains impaired this would advocate studying the role of CGT and cholesteryl glucosides both during the early stages of infection establishment, and during the persistence stage, to identify which virulence mechanisms are impeded.

The conditional strains generated in this study match wild-type CGT expression levels in the repressed state and can be used to investigate the effects fluctuations in CGT expression have, within the same strain, on bacterial-host interactions using *in vitro* cell culture models. To distinguish between the biological functions of cholesteryl glucosides and free cholesterol in the bacteria during infection, CGT over expression may provide a mechanism by which free cholesterol can be depleted in the bacterial membrane without inhibiting cholesteryl glucoside biosynthesis. Such an approach would be more selective than experiments in which cholesterol is depleted in bacterial membranes by limiting cholesterol in the growth media or by incubation with β-cyclodextrins, as these approaches result in reduction in both cholesterol and cholesteryl glucosides (Wunder et al., 2006). In addition, this study has demonstrated that strains expressing CGT labelled with a C-terminal peptide tag can synthesize cholesteryl glucosides and colonize mice efficiently. These strains are useful tools for future *in vitro* and *in vivo* studies aimed at imaging CGT localization during different infection or growth states. Antibodies specific for CGT would be more ideal however, and therefore their production will be undertaken in future studies. Production of antibodies against specific CGT peptides has recently been described, but only one of the three antibodies produced could detect CGT in *H. pylori* lysates and it was specific to strain 26695 (Hoshino et al., 2011).
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The recent evidence that *H. pylori cgt* knockouts can in fact colonize animal hosts means that any future studies using conditional *cgt* mutants in the animal model will have to be carefully designed. Though the utility of conditional *cgt* mutants is now more limited in light of the experiments conducted here, the attempt to generate such a mutant has revealed the necessity to improve upon the *uPtetO* based tet-system in *H. pylori* in order to efficiently regulate *cgt* and other genes with similarly low levels of expression.

Reduction of residual transcription from tet-promoters has often been achieved by reducing the promoter strength through the incorporation of a second tetO site, which consequently also reduced the maximal amount of gene induction (Geissendorfer and Hillen, 1990; Lutz and Bujard, 1997). The addition of a second and third tetO site decreased *cgt* induction but did not result in a significant decrease in residual *cgt* transcription from either *uPtetO2* or *uPtetO3*. There are several additional approaches for reducing residual transcription in *H. pylori* tet-promoters that can be explored. These would involve:

- Mutation of the *E. coli* canonical -10 region in *uPtetO* from (TATAAT) back to the original *ureA* promoter -10 (TACAAT) (Davies et al., 2002) or to a less efficient sequence to reduce initiation of transcription by RNA polymerase (Knaus and Bujard, 1990).
- Mutation of the Shine-Dalgarno sequence to decrease binding of the mRNA transcript by the ribosome machinery (Ringquist et al., 1992).
- Mutation of the ATG start codon to a rare alternative start codon to stall initiation of translation and protein synthesis (Ringquist et al., 1992).
- Increased expression of TetR by introducing a second copy of *tetR* in the genome (Guo et al., 2007; Kamionka et al., 2005).
- Testing the functionality of the P_LtetO-1 promoter in *H. pylori*. This promoter has been optimized for tight repression in *E. coli* and been demonstrated to work in several different species of γ-proteobacteria (Hsiao et al., 2006; Lathem et al., 2007; Lutz and Bujard, 1997; Qian and Pan, 2002).

Should P_LtetO-1 prove to be a very weak promoter *H. pylori*, it is highly likely that it will be silenced very efficiently and therefore would be optimal for tight regulation of low levels of gene expression. Such a strategy worked very well for the lacP-based repressor system developed for *H. pylori* (Boneca et al., 2008). The P_tac promoter, whose regulation had been previously optimized in *E. coli*, was a disappointingly weak...
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promoter in *H. pylori*, however it was repressed very efficiently and thus was ideal for generating conditional mutants of essential genes in *H. pylori*.

A sensitive reporter gene will also be required in order to test improved repression of new *H. pylori* tet-promoters. The *lacZ* gene encoding β-galactosidase could be used as it served as a suitable reporter to confirm tight regulation of the *lacI*<sup>q</sup>-based repressor system (Boneca et al., 2008) although more sensitive reporters such as one of the *luciferase* gene variants, like the small thermostable MpLuc1 from *Metridia pacifica* (Takenaka et al., 2008), should be explored to translate additional molecular tools from *E. coli* to the study of *H. pylori*. Though TLC analysis of CGT activity on NBD-cholesterol is only qualitative it could also serve as a reporter for improved promoter repression in *H. pylori*. This analysis could be expanded to a quantitative assay using high-performance liquid chromatography (HPLC), but the synthesis of the NBD-cholesteryl glucoside standards would need to be achieved.

Although the final aims of this study were not achieved, this study identified that CGT is not abundantly expressed and that perturbations in CGT expression affect the ability of *H. pylori* to establish infection. This study confirmed that the *uPtetO* based tet-system can be used to complement and regulate *H. pylori* genes, however the system is currently limited to more abundant gene products and modifications need to be made to make the system suitable for regulating gene products that are found in very low abundance.
6 Tet-regulation of urease: a H. pylori virulence factor essential for colonization

6.1 Introduction

H. pylori is highly adapted to colonizing and surviving in the harsh conditions of the gastric environment. One of its key adaptations is the virulence factor urease. This enzyme is abundantly expressed by H. pylori, at levels exceeding that of any other known microbe (Mobley, 2001), and is estimated to constitute 10-15% of the bacterium’s total protein content (Bauerfeind et al., 1997). Urease is essential for colonization as H. pylori urease mutants are unable to establish infection (Eaton and Krakowka, 1994; Tsuda et al., 1994a; Tsuda et al., 1994b; Wirth et al., 1998).

A majority of the urease expressed by H. pylori is localized in the bacterial cytoplasm, although up to 10% may be located on the cell surface due to either cell lysis (Bode et al., 1993; Phadnis et al., 1996) or specific export (Gobert et al., 2002b; Vanet and Labigne, 1998). Urease is a multimeric enzyme, consisting of 12 UreA and UreB heterodimers, and requires Ni²⁺ as a cofactor for catalytic activity (Ha et al., 2001; Hawtin et al., 1991; Hu et al., 1992). The substrate for urease is the molecule urea. Urease catalyses the hydrolysis of urea to produce CO₂ and NH₃, which acts to buffer the acidity of the local environment around the cell. Of note is that H. pylori urease has an optimal catalytic activity within a pH range of 7.5 to 8.0. Its activity greatly decreases at pH 5.0 and it is irreversibly inactivated at pH 4.0 (Scott et al., 1998).

The high, but regulated, activity of H. pylori urease allows the bacterium to resist the highly acidic environment of the stomach (Figure 6-1). All aerobic bacteria, including H. pylori, require a constant proton motive force (PMF) for the synthesis of ATP, by way of the F₁F₀ ATP synthase, and the function of membrane transporters. The PMF is the sum of the pH gradient and the membrane potential across the inner membrane (Sachs et al., 2003). In the absence of urea, acidification of the environment around H. pylori increases the pH gradient across its inner membrane, and consequently the membrane potential decreases to maintain a stable PMF. However, when the pH of the environment decreases to 3.5, membrane potential is irreversibly lost and H. pylori dies (Meyer-Rosberg et al., 1996; Scott et al., 1998). When urea is present in the
environment, it is hydrolyzed by urease to produce NH₃ which can freely diffuse out from the cytoplasm and elevate the pH in periplasm to reduce the pH gradient across the inner membrane. However this process must be controlled as unregulated cytoplasmic urease activity at neutral pH would lead to lethal alkalinisation due to membrane potential collapse at pH 8.4 (Scott et al., 1998). The pH of the stomach can fluctuate significantly, rising to neutral due to the buffering action of food during digestion and falling to as low as pH 1.0 in the absence of food (Sachs et al., 2003). Therefore to regulate urease activity in such an unstable environment, the uptake of urea into the bacterium’s cytoplasm is controlled by an acid-gated urea channel, UreI, that does not open until the periplasmic pH decreases below pH 6.5 (Scott et al., 2000; Weeks et al., 2000; Weeks et al., 2004). In this way, urease can only act on urea, to buffer the pH of the periplasm, when the bacterium is in an acidic environment. This process of pH regulation, mediated by urease and UreI, maintains PMF over a relatively wide range of gastric pH, permitting *H. pylori* (a neutrophile) to grow and maintain protein synthesis in an acidic environment (Scott et al., 1998).

**Figure 6-1  Model of *H. pylori* urease mediating acid resistance**

The inner and outer membrane is separated by the periplasmic space. PMF, required for ATP synthesis by F₁F₀ ATP synthase (purple structure) and membrane transport, is dependent on both the pH gradient across the inner membrane and membrane potential (PD). Urea transport into the cytoplasm is regulated by UreI, an acid-gated urea channel (blue cylinder). Cytoplasmic urease is able to compensate for environment acidity by the hydrolysis of urea when the external pH decreases to < 6.5. This enables the buffering of the periplasmic space to about pH 6.2, which in turn provides a stable PD over a relatively wide range of gastric pH encountered by *H. pylori*. Active urease (orange oval cluster) and urease apoenzyme (yellow oval cluster). (Adapted from (Scott et al., 1998))
As *H. pylori* resides deep within the gastric mucus and on the epithelial surface, where the pH is greater than 5.5 (Blaser and Kirschner, 1999; Schreiber et al., 2004), and neutralization of gastric pH fails to restore colonization by urease mutants (Eaton and Krakowka, 1994), it is thought that urease has additional roles during infection other than simply neutralizing acid in the gastric lumen. A significant amount of urease is found on the bacterial cell surface (~10% of total urease protein) due to either cell lysis (Marcus and Scott, 2001; Phadnis et al., 1996) or specific export (Gobert et al., 2002b; Vanet and Labigne, 1998). The biological significance of surface urease is unclear. There is significant debate whether surface urease also mediates acid resistance. Inhibition of surface urease in young (< 24 h) *H. pylori* cultures was found to lead to acid sensitivity (Krishnamurthy et al., 1998), however urease activity is essentially non-existent at pH 5 (Scott et al., 1998) and only wild-type strains survive when animal models are co-infected with both wild-type and urease mutant *H. pylori*, suggesting that urease released by wild-type bacteria is not sufficient to protect the urease mutants (Eaton and Krakowka, 1994; Sachs et al., 2003). Thus it is unlikely that surface urease has any significant role in acid resistance however it may have an alternative role in pathogenesis. The products of urea hydrolysis have been shown to directly contribute to virulence. NH$_3$ produced by urease activity causes damage to gastric epithelium by disrupting tight cell junction integrity (Lytton et al., 2005; Wroblewski et al., 2009) and CO$_2$ protects against the bactericidal activity of the nitric oxide metabolite, peroxinitrite, produced by phagocytes to kill engulfed bacteria. (Kuwahara et al., 2000) 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 and induce cell apoptosis (Fan et al., 2000) and the UreB subunit has been shown to activate monocytes and stimulate them to release proinflammatory cytokines by binding to cell surface CD74, a MHC class II associated invariant chain (Beswick et al., 2006; Harris et al., 1996). In addition, changes to the surface of the urease complex resulted in the eventual clearance of *H. pylori* infection in mice (Schoep et al., 2010). Loss of colonization was attributed to the disruption in urease mediated interactions between *H. pylori* and host cells as urease activity was unaffected by the surface mutation, ruling out loss of acid resistance or nitrogen assimilation (Williams et al., 1996) as contributing factors (Schoep et al., 2010). Together, all these observations seem to suggest that urease found on the surface is important in the interactions between *H. pylori* and host cells.
Since urease is essential for colonization, it is difficult to directly study its function \textit{in vivo} and its role in persistence using conventional urease knockout mutants. The availability of a conditional urease mutant would overcome this constraint and permit changes to the urease phenotype during an established infection. To conclusively determine if urease is required by \textit{H. pylori} once infection is established, when the bacterium has reached the relatively neutral environment of its gastric niche, and to investigate the potential immune modulatory role of urease \textit{in vivo}, conditional urease \textit{H. pylori} mutants were generated using the \textit{tet}-system developed for \textit{H. pylori} (Chapter 4).

The urease genes are transcribed from two operons (Figure 6-2 A). The first operon consists of \textit{ureA} and \textit{ureB} which encode for the 27 kDa UreA and 62 kDa UreB urease subunits respectively. The second operon consists of \textit{ureI}, \textit{ureE}, \textit{ureF}, \textit{ureG} and \textit{ureH}. The last four genes encode accessory proteins required for assembly of the urease apoenzyme and Ni\textsuperscript{2+} incorporation to produce catalytically active urease (Mobley, 2001). In this study, a \textit{H. pylori} conditional urease mutant was generated by placing the expression of the urease subunits, UreA and UreB, under \textit{tet}-control. To achieve this, sequences within the \textit{ureA} promoter, \textit{P}$_{ureA}$, were replaced with \textit{tetO} to generate several derivatives of \textit{P}$_{ureA}$, \textit{urePtetO}(I-V). \textit{P}$_{ureA}$ has strong constitutive basal levels of transcription which can be further increased in response to fluctuations in environmental pH and free Ni\textsuperscript{2+} concentration (Contreras et al., 2003; Pflock et al., 2006). Induction of \textit{P}$_{ureA}$ is mediated by the NikR and ArsRS systems, whose regulatory sequences are located upstream of the \textit{P}$_{ureA}$ -10 region (Figure 6-2 B). Conversion of \textit{P}$_{ureA}$ to a \textit{tet}-responsive promoter, \textit{urePtetO}, did not affect NikR operator sequences (Delany et al., 2005), however \textit{tetO} sequences were introduced within the downstream ArsR\textsim P binding region (Pflock et al., 2005) and therefore may have introduced mutations in the ArsR\textsim P operator sequence.

Several promoter architectures were designed to regulate the \textit{ureAB} operon and they were evaluated in \textit{H. pylori} X47 strains expressing TetRs (Chapter 4) for \textit{tet}-regulated urease expression and activity. Conditional urease mutants were used to study the role of this essential colonization factor in infection maintenance and immune modulation \textit{in vivo}. 

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Figure 6-2  Schematic diagram of the urease locus and the ureA promoter

A. The genes that comprise the urease locus are depicted. Genes ureA and ureB encode the urease structural subunits, gene ureI encodes the acid-gated urea channel and genes ureE-H encode the urease accessory proteins. The size of each ORF is indicated below (bp). Transcription occurs from two promoters, P_{ureA} and P_{ureI}, indicated by the red arrows.

B. Diagram of the ureA promoter. The transcriptional start point (TSP) is indicated by the black arrow. The white boxes denote the -10, the extended -10 and -35 regions. Green shaded box indicates NikR binding region identified by DNase I protection assay and grey boxes denote NikR operator sequence identified by mutational analysis (Delany et al., 2005). Two blue shaded boxes indicate the binding regions of the response regulator ArsR~P identified by DNase I protection assay. The ArsR~P operator sequences have not yet been described. Grey numbers on either side of the shaded boxes indicate the nucleotide region protected in DNase I protection assays. Red bars indicate promoter sequences altered by tetO replacement in tet-responsive P_{ure} derivatives, urePtetO.
6.2 Aims

This section of the study addressed the following aims.

1. Generate additional tetracycline responsive *H. pylori* promoter constructs.
2. Regulate *ureA* and *ureB* expression *in vitro* and *in vivo* using *tet*-repressor system.
3. Characterize changes in host immune response upon down regulation of urease expression after infection has been established.
6.3 Methods

6.3.1 In vitro characterization of urePtetO regulation

6.3.1.1 Urease plate assays

_Urease culture plates:_ Brucella broth, 7% NCS, 1 mM urea, phenol red 100 mg/l, vancomycin 6 mg/l, pH ~ 6.2. The pH of the plate 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.

_Screen for 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 urease plates and incubated under microaerobic conditions. Urease 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. Tet-ON strains grown on CBA plates without ATc remained urease negative, while strains grown on CBA plates with ATc became urease positive. Conversely, for Tet-OFF strains, _H. pylori_ clones grown on CBA plates without ATc were urease positive and clones grown on CBA plates with ATc became urease negative.

_Induction of urease activity in Tet-ON X47 mdaB::ptetR; urePtetO strains:_ Tet-ON strains grown on CBA plates were plated onto urease culture plates. Blank discs loaded with 10 μl of ATc solution were placed onto the inoculated plate and incubated under microaerobic conditions. Plates were examined for colour change every 20 h. Alternatively, Tet-ON strains grown on CBA plates were resuspended in PBS to OD₆₀₀ = 0.1. A 20 μl aliquot of this bacterial suspension was spotted onto fresh urease plates containing ATc in concentrations ranging from 0 to 250 ng/ml. Plates were incubated under microaerobic conditions and examined for colour change every 24 h.
6.3.1.2 Measurement of urease activity

The urease activity assay routinely used in the laboratory was developed by Ondek Pty. Ltd. (Dr. Tobias Schoep) and is originally adapted from the protocol previously described (Hamilton-Miller and Gargan, 1979). Strains were grown on CBA plates with or without 50 ng/ml ATc for two successive passages. Bacteria were collected and resuspended in cold buffer A (25 mM phosphate buffer, pH 6.8) and standardized to an OD<sub>600</sub> = 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.

6.3.1.3 Immunoblot analysis of ATc regulated UreB expression

Bacteria were cultured on CBA plates with or without 50 ng/ml ATc for 48 h before they were collected, resuspended in PBS, washed twice and finally resuspended in Tris lysis buffer. Whole cell lysates were prepared as described (section 2.2.4.1). Samples were analysed for expression of the UreB subunit of urease by immunodetection as described (sections 2.2.4.2 and 2.2.4.3). PVDF membranes were probed with an Anti-UreB antibody (1:8000) followed by incubation with Rabbit Anti-mouse IgG HRP conjugate antibody (1:10000).

For time course experiments, analysing <i>tet</i>-regulated induction and repression of UreB expression, the following protocol was used. <i>H. pylori</i> cultures were grown for 14 h to mid-log phase in 10 ml of HI medium. An aliquot of 2 ml was taken from each culture and used as time 0, and an 8 ml aliquot of fresh media containing 400 ng/ml ATc was added to each culture to give a final volume of 16 ml and 200 ng/ml ATc. The bacteria were incubated for another 30 h, with aliquots taken from each culture at 2, 4, 8, 16, 24 and 30 h after induction with ATc. Bacteria were harvested by centrifugation, washed twice with PBS and finally resuspended in Tris lysis buffer. Samples were analysed as described above.
6.3.2 Animal Experiments

Robyn Himbeck, Michelle Middelton and Tania Camilleri provided technical assistance with the animal experiments. They performed the retro orbital and cardiac bleeds, and were involved with challenging mice with X47 strains and helped to process mouse stomachs for evaluation of bacterial load.

6.3.2.1 Screening of *H. pylori* strains X47 *urePtetO*(I-V) for restored colonization.

Mice, n = 3 per group, were orally challenged with X47 *urePtetO*(I-V) strains (Table 3-2, hAD177-hAD181). Mice were sacrificed two weeks after oral challenge and the bacteria load was evaluated as described (section 2.3).

6.3.2.2 Colonization by *tet*-responsive conditional urease mutant strains

Tet-ON conditional urease mutant strains were passaged on CBA plates containing 50 ng/ml ATc for 48 h and then harvested and prepared as bacterial inoculums. Mice were supplemented as indicated with Dox or ATc in a 5% sucrose solution. Mice were sacrificed one or two weeks after oral challenge and the bacteria load was evaluated.

6.3.3 Characterization of immune response

These experiments were designed and conducted in collaboration with Dr. Senta Walton. Robyn Himbeck and Tania Camilleri provided technical assistance with the experiments including, oral challenge, isolation of gastric lymph nodes, cardiac and retro orbital bleeds and stomach processing.

6.3.3.1 Collection of tissue samples

*Stomachs:* The stomach was removed and placed in ice-cold PBS to prevent dehydration. The fore stomach and stomach content were discarded and remaining tissue was rinsed in HI broth. Using a 27½ G syringe needle, infiltrating lymphocytes were flushed from the submucosa by repeated injection of PBS into the gastric wall to a total volume of 10 ml (Martinelli et al., 1996). The lymphocytes from 5 mouse stomachs were pooled, forming two samples for each treatment group of 10 animals, and collected by centrifugation at 1600 rpm for 5 min. The cell pellet was resuspended in 0.3 ml of FACS buffer. The remaining stomach tissue was homogenized in the HI
broth used to rinse the stomach. An aliquot of this homogenate was used to prepare serial dilutions which were plated onto CBA plates to determine the bacterial load for each animal. The remaining stomach homogenate was clarified by centrifugation at 20,000 g for 5 min. The clarified supernatant was transferred to a fresh tube and stored at -20 °C.

**Gastric lymph nodes and spleens:** The gastric lymph node (gLN) (Figure 6-3) and spleen (of a naive animal) was removed and placed in ice-cold PBS to prevent dehydration. These tissues were pressed through a cell strainer to obtain a single cell suspension. Isolated cells were pelleted by centrifugation at 1600 rpm for 5 min and resuspended in 0.3 ml (gLN) or 3 ml (spleen) of FACS buffer.

**Sera:** Animals were anesthetized using isoflurane. Blood was collected either by retro orbital bleed on one eye during the study or by cardiac bleed at the experiment end point. Blood was transferred to blood collection microtubes and sera were separated from blood cells by centrifugation at 6,000 g for 10 min. Mouse sera were stored at -20 °C.

### 6.3.3.2 ELISA

**Detection of anti-*Helicobacter pylori* antibodies in serum**

MaxiSorp® 96 well plates (Nunc) were coated with X47 antigen by incubating plates at 4 °C overnight with X47 whole cell lysate (1 μg/well) diluted in coating buffer (0.5 M NaHCO3, pH 9.6). Plates were washed 5 times with washing buffer (PBS, 0.05% Tween-20) and incubated with blocking buffer (PBS, 2% BSA) at 37 °C for 120 min. Plates were washed twice with washing buffer and incubated with serum samples diluted 1:20 with dilution buffer (PBS, 0.05% Tween-20, 2% BSA) at room temperature for 60 min. Plates were washed 5 times with wash buffer and incubated with Alkaline Phosphatase conjugated Sheep Anti-mouse IgG at a dilution of 1:1000 at room temperature for 60 min. Plates were washed again and incubated with substrate solution (0.2 M Tris, pNPP 1 mg/ml) in the dark. The reaction was stopped after 40 min with 2 M NaOH and the absorbance at 405 nm was measured. As a positive control, Rabbit Anti-*Helicobacter pylori* was used at a dilution of 1:1000, followed by incubation with Alkaline Phosphatase conjugated Goat Anti-rabbit IgG at a dilution of 1:1000.
Figure 6-3  Location of mouse gastric lymph node

A. Photograph of the mouse abdominal cavity with stomach (a), liver (b), and spleen (c). Black arrow indicates position of gastric lymph node (gLN), an unpaired lymph node in the lesser omentum at the minor curvature of the stomach. White arrow indicates position of the Pancreaticoduodenal lymph node, an unpaired lymph node in the mesoduodenum, dorsal to the portal vein, surrounded by pancreatic tissue.

B. Schematic diagram depicting the localization of lymph nodes in the mouse. Position of gLN (13) is indicated.

These figures were taken from a paper by Van den Broeck, et al. describing the anatomy and nomenclature of murine lymph nodes. (Van den Broeck et al., 2006)

Detection of anti-Helicobacter pylori antibodies in stomach homogenate
A similar protocol to the one described above was used to measure anti-\textit{Helicobacter} IgA in clarified stomach homogenate solution. Due to the stomach flushing procedure used to isolate host immune cells (6.3.3.1), X47 antigen coated the ELISA plates were incubated with neat dilutions of clarified stomach homogenate. IgA antibodies were detected using Alkaline Phosphatase conjugated Goat Anti-mouse IgA at a dilution of 1:1000 at room temperature for 60 min. After the final wash, plates were incubated with substrate solution (0.2 M Tris, pNPP 1 mg/ml) in the dark. The reaction was stopped after 160 min with 2 M NaOH and the absorbance at 405 nm was measured.
6.3.3.3 Flow cytometry staining and analysis

ACK buffer
150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.4

FACS buffer
1× PBS containing 2% FCS (v/v), 10 mM Na₂EDTA, 0.09% NaN₃ (w/v)

Staining protocols
A final suspension of lymphocytes isolated from mouse stomachs, gLNs or spleen (6.3.3.1) was made in FACS buffer and aliquoted into the wells of a 96 well plate for fluorescent antibody staining.

Staining A – Stain for innate immune cells
Cells in a 100 μl cell suspension were pelleted by centrifugation at 1600 rpm for 5 min. The supernatant was decanted and cells were resuspended in 50 μl of surface antibody mix (FACS buffer, CD11b-APC 1:250, CD11c-PE 1:100, Ly6C-PerCP-Cy5.5 1:250, Ly6G-APC-Cy7 1:100, 1-A(b)-Biotin 1:100, Streptavidin-PE-Cy7 1:250, CD117-V450 1:250, LIVE/DEAD aqua 1:500) and incubated at 4 °C for 20 min. A 150 μl aliquot of ACK buffer was added and cells were incubated at room temperature for 5 min. Cells were finally washed with FACS buffer and fixed in 50 μl of 1× BD stabilizing fixative solution.

Staining B – Stain for adaptive immune cells
Cells in a 100 μl cell suspension were pelleted by centrifugation at 1600 rpm for 5 min. The supernatant was decanted and cells were resuspended in 50 μl of surface antibody mix (FACS buffer, CD3-PE 1:100, CD4-Pe-Cy7 1:250, CD8a-PerCP-Cy5.5 1:100, CD19-APC 1:250, CD45.2-V450 1:250, LIVE/DEAD aqua 1:500) and incubated at 4 °C for 20 min. A 150 μl aliquot of ACK buffer was added and cells were incubated at room temperature for 5 min. Cells were finally washed with FACS buffer and fixed in 50 μl of 1× BD stabilizing fixative solution.

Multiparameter flow cytometric analysis was performed using a FACS Canto II flow cytometer (BD, Bioscience) with FACS DIVA software (BD, Bioscience). Data were analyzed using FlowJo software (Treestar, San Carlos, CA).
6.4 Results

To generate conditional urease mutants, the native ureA promoter, P_{ureA}, was transformed into a tetracycline responsive promoter. Modifications to P_{ureA} were based on the strategy used to generate the uPtetO tet-responsive promoters. In addition to the promoter architectures studied in Chapter 4, two new promoter architectures were investigated for tet-regulation in H. pylori. Wild-type nucleotide sequences flanking the -35 and -10 promoter regions of P_{ureA} were replaced with tetO sequences to generate several derivatives of P_{ureA}, uPtetO(I-V) (Figure 6-4). These promoter constructs were used to replace the native urease promoter in strain X47 and were assessed for their ability to regulate urease expression.

6.4.1 Characterization of uPtetO promoter strength

The urease activities of H. pylori strains harbouring one or more tetO sites in the ureA promoter, strains X47 uPtetO(I-V) (Table 3-2, hAD177-hAD181), were measured and compared to the wild-type X47 strain (Figure 6-5 A). The urease activity in strains harbouring uPtetOI, II and V was similar to that of wild-type, while urease activity in strains with uPtetOIII and uPtetOIV was only 40% and 75% of wild-type activity respectively. The amount of total urease protein expressed by X47 uPtetO strains was analyzed by immunodetection of the UreB subunit in total H. pylori cell lysates (Figure 6-5 B). X47 strains uPtetOI, II and V expressed approximately equal amounts of UreB but expression was less than that of wild-type X47. Strains with uPtetOIII and uPtetOIV expressed significantly reduced amounts of UreB, with strain X47 uPtetOIII expressing the least of all the uPtetO strains. To assess what level of urease expression was required for colonization, the X47 uPtetO strains were evaluated for their ability to colonize C57BL/6J mice (Figure 6-5 C). All five X47 uPtetO strains were successfully re-isolated from mouse stomachs two weeks after oral challenge. The bacterial load was reduced in strains X47 uPtetOI, X47 uPtetOIII and X47 uPtetOIV, however the infection rate of X47 uPtetOI was the same as X47 uPtetOII, X47 uPtetOV and the wild-type X47 strain. Sequencing of the ureA promoter region of re-isolated X47 uPtetO strains revealed that all uPtetO sequences remained unchanged after passage through mice.
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Figure 6-4  Schematic diagram of tetracycline responsive promoters, *urePtetO*

A. Nucleotide sequence (partial) of the wild-type *ureA* promoter, *P*<sub>ureA</sub>, and tetracycline responsive *P*<sub>ureA</sub> 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*<sub>ureA</sub> promoter sequence has been replaced with *tetO* sequences.
6.4.2 \textit{Tet-regulation of urease expression in H. pylori.}

As all the X47 \textit{urePtetO} strains were able to colonize the mouse model, all five promoter derivatives were used to generate a set of 33 different conditional X47 urease mutants (Table 3-7, hAD189-hAD221). These X47 \textit{ptetR; urePtetO} strains expressed either TetR or revTetR, and expressed \textit{ureA} and \textit{ureB} under the control of a modified \textit{ureA} promoter containing one or more \textit{tetO} sites. \textit{H. pylori} strains expressing revTetR and transformed with \textit{urePtetO} were designated as Tet-OFF strains as, while urease positive under standard growing conditions, they were expected to become urease negative in the presence of tetracycline. Conversely, \textit{H. pylori} strains expressing TetR and transformed with \textit{urePtetO} were designated as Tet-ON strains as they were urease negative under standard growing conditions and were expected to become urease positive only in the presence of tetracycline.

6.4.2.1 Characterization of \textit{urePtetO} regulated UreB expression and urease activity \textit{in vitro}

Regulation of \textit{urePtetO} promoters by ATc was analyzed by immunodetection of the UreB protein subunit and by measuring urease activity. Under standard growing conditions, the amount of UreB protein in strains expressing TetR (Tet-ON strains) was below the level of detection but was significantly increased when strains were grown in the presence of 50 ng/ml ATc (Figure 6-6). Induced UreB expression was greatest for strains with \textit{urePtetO} constructs I, II and V. Analysis of UreB protein in strains expressing revTetR (Tet-OFF strains) revealed that under standard growing conditions, promoter constructs \textit{urePtetOIII} and IV were the weakest of the five \textit{urePtetO} promoters (Figure 6-7 A). These weaker promoters could be fully repressed when strains were grown in the presence of 50 ng/ml however at this concentration silencing of \textit{urePtetOI}, II and V was incomplete. Higher concentrations of ATc were tested and UreB protein levels could be reduced below the detection limit in Tet-OFF strains using 300 ng/ml ATc (Figure 6-7 B).
Figure 6-5  Urease activity and expression in X47 ureP tetO(I-V) strains
Figure 6-5  Urease activity and expression in X47 urePtetO(I-V) strains
(adjacent page)

A. Urease activity in X47 urePtetO(I-V) strains compared to wild-type X47. Fresh
cultures of H. pylori grown on CBA plates were used in the assay (section
5.3.4.2) 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.

B. UreB expression in X47 urePtetO(I-V) strains compared to wild-type X47.
Fresh cultures of H. pylori grown on CBA plates were used to prepare H. pylori
lysates. Equal amount of protein (~15 μg) was loaded into each lane and
separated on a 10% SDS–PAGE gel. Data are representative of three
independent experiments.
Lane 1, X47 urePtetOI
Lane 2, X47 urePtetOII
Lane 3, X47 urePtetOIII
Lane 4, X47 urePtetOIV
Lane 5, X47 urePtetOV
Lane 6, wild-type X47

C. 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 mean bacterial load per
group (n = 3) and points plotted represent colonization density for each
individual animal. Detection limit was < 20 CFU per stomach (dotted horizontal
line). Gastric specimens without H. pylori re-isolation are shown as null.
Figure 6-6  Tet-regulation of urePtetO in H. pylori Tet-ON strains

UreB protein was detected in H. pylori strains harbouring urePtetO and expressing TetR (ptetR2, ptetR4 and ptetR6). Bacteria were cultured in the absence or presence of 50 ng/ml ATc for 48 h and fresh bacteria cultures were used to prepare whole cell lysates. Equal amount of protein (~15 μg) was loaded into each lane and separated on a 10% SDS–PAGE gel. 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, II and V. UreB expression was weaker in strains harbouring urePtetOIII and IV.
Figure 6-7  *Tet*-regulation of *urePtetO* in *H. pylori* Tet-OFF strains

UreB protein was detected in *H. pylori* strains harbouring *urePtetO* and expressing revTetR (*ptetR5* and *ptetR7*). Bacteria were cultured in the absence or presence of ATc for 48 h and fresh bacteria cultures were used to prepare whole cell lysates. Equal amount of protein (~15 μg) was loaded into each lane and separated on a 10% SDS–PAGE gel. The *urePtetO* construct is specified under the bars. (A) Strains grown in the presence of 50 ng/ml ATc. UreB protein could not be detected in strains harbouring *urePtetO* III and IV when grown in the presence of 50 ng/ml ATc but could be detected, although at significantly reduced levels, in strains harbouring *urePtetO* I, II and V. (B) Strains grown in the presence of 300 ng/ml ATc. Complete repression of UreB expression was achieved in strains harbouring *urePtetO* I, II and V using higher ATc concentrations. High ATc concentration did not alter UreB expression in wild-type X47 strain (WT).
The urease activity of Tet-ON and Tet-OFF strains relative to wild-type X47 was measured using an assay developed by Ondek Pty. Ltd. (Dr. Tobias Schoep). In this study, the detection limit of the assay was the activity of 0.5 U of Type III urease from Jack bean (Sigma, USA). Under standard growing conditions, the urease activity of Tet-ON strains was below the detection limit of the assay (Figure 6-8 A-C). When grown in the presence of 50 ng/ml ATc, urease activity in strains ptetR2; urePtetOII (Figure 6-8 A), and ptetR4; urePtetOI, II and V (Figure 6-8 B) was induced to wild-type levels of activity, while urease activity was only partially induced in strains: ptetR2; urePtetOI and V (Figure 6-8 A) and ptetR6; urePtetOV (Figure 6-8 C). For the remaining Tet-ON strains, induced urease activity was below 10% of wild-type urease activity.

The urease activity of Tet-OFF strains under standard growing conditions for strains transformed with urePtetO constructs I, II and V was equal to, or slightly greater than wild-type, while the urease activity of strains transformed with urePtetO constructs III and IV was 60-70% of wild-type activity (Figure 6-9). Urease activities of Tet-OFF strains grown in the presence of 50 ng/ml ATc were between 35% and 10% of wild-type X47 urease activity and were below the limit of detection when Tet-OFF strains were grown in the presence of 300 ng/ml ATc. For X47 strains transformed with urePtetO and either ptetR1 or ptetR3 (Table 3-7, hAD189-hAD193, hAD199-hAD203), UreB expression could not be significantly reduced using 50 ng/ml ATc based on their urease activities. Therefore complete characterization of tet-regulated urease expression was not conducted on these strains.
Tet-ON strains

A. X47 strains transformed with \textit{ptetR2} (P\textsubscript{amiE-tetR}) and \textit{urePtetO(I-V)}
B. X47 strains transformed with \textit{ptetR4} (P\textsubscript{flaA-tetR}) and \textit{urePtetO(I-V)}
C. X47 strains transformed with \textit{ptetR6} (P\textsubscript{Taat-tetR}) and \textit{urePtetO(III-V)}

All measurements were carried out in triplicate. Data are averages of three independent experiments and error bars represent the standard deviation.

Figure 6-8  Tet-regulation of urease activity in \textit{H. pylori} Tet-ON strains

Urease activity assays of Tet-ON strains. Bacteria were cultured in the absence (white bars) or presence of 50 ng/ml ATc (black bars) for 48 h and fresh bacteria cultures were used for activity assays. Urease activity is expressed as a percentage of wild-type X47 grown under standard conditions (WT).

A. X47 strains transformed with \textit{ptetR2} (P\textsubscript{amiE-tetR}) and \textit{urePtetO(I-V)}
B. X47 strains transformed with \textit{ptetR4} (P\textsubscript{flaA-tetR}) and \textit{urePtetO(I-V)}
C. X47 strains transformed with \textit{ptetR6} (P\textsubscript{Taat-tetR}) and \textit{urePtetO(III-V)}
Figure 6-9  
*Tet*-regulation of urease activity in *H. pylori* Tet-OFF strains

Urease activity assays of Tet-OFF strains. Bacteria were cultured in the absence (white bars) or presence of either 50 ng/ml ATc (grey bars) or 300 ng/ml ATc (black bars) for 48 h and fresh bacteria cultures were used for activity assays. Urease activity is expressed as a percentage of wild-type X47 grown under standard conditions (WT).

A. X47 strains transformed with *ptetR* (P_{taTaat}-*revtetR*) and *urePtetO*(I-V)

B. X47 strains transformed with *ptetR7* (P_{taCaat}-*revtetR*) and *urePtetO*(I-V)

All measurements were carried out in triplicate. Data are averages of three independent experiments and error bars represent the standard deviation.
6.4.2.2 **Induction of urePtetO is dose- and time-dependent**

The *tet*-regulated urease expression in Tet-ON strains was further characterized by assaying urease activity under increasing inducer concentrations and by evaluating UreB expression at several different time points.

A sensitive urease plate assay was used to detect residual urease activity and low levels of *urePtetO* induction in Tet-ON strains. A disc diffusion assay using acidified urease plates was conducted to demonstrate that induction of urease activity was ATc dependent. Urease plates were inoculated with urease negative X47 *ureA::rpsL-cat* strain or conditional X47 urease mutant strains that were either untreated or grown with 50 ng/ml ATc for 48 h prior to testing. Discs containing ATc were placed onto each inoculated urease plate and incubated under microaerobic conditions. Urease plates inoculated with an ATc pre-induced Tet-ON strain changed colour within 30 min of inoculation (Figure 6-10 A). For untreated conditional X47 urease mutants, urease activity was only evident after 20 h and was confined to the regions around the discs containing ATc (Figure 6-10 B). Urease plates inoculated with a urease negative X47 *ureA::rpsL-cat* strain remained yellow for the entire experiment (Figure 6-10 C), demonstrating that the observed colour change in the urease plates was due to *H. pylori* urease activity and not due to other metabolic processes.

Tet-ON strains grown on acidified urease plates containing different concentrations of ATc demonstrated that all the *urePtetO* promoters could be induced to some extent and that induction was dependent on ATc concentration. For *ptetR*4 strains (Figure 6-11), the lowest concentration tested, 1 ng/ml of ATc, was sufficient to induce maximal urease activity in all five *urePtetO* promoters. Higher concentrations of ATc did not result in increased activity; instead urease activity decreased at concentrations above 25 ng/ml and bacterial growth was reduced, suggesting that ATc may be more toxic to *H. pylori* in acidified Brucella broth media compared to neutral media (Figure 4-7). For *ptetR*6 strains (Figure 6-12), significant urease activity was detected after 24 h at ATc concentrations of 5 ng/ml and 10 ng/ml for *urePtetOV* and *urePtetOIV* transformed strains respectively. Urease activity due to induction of *urePtetOIII* was only evident after 48 h at 25 ng/ml and 50 ng/ml ATc. As observed for *ptetR*4 Tet-ON strains, urease activities decreased in *ptetR*6 Tet-ON strains at concentrations above 25 ng/ml ATc. Furthermore, for all Tet-ON strains tested, very low levels of urease activity were
detected after 48 h on plates without ATc (Figure 6-11 and 6-12), which suggests that there was residual transcription of *ureA* and *ureB* from repressed *urePtetO* promoters.

**Figure 6-10  Tet-regulation of urease activity on acidified urease plates**

A. Strain X47 *ptetR2; urePtetOV* (hAD198) was passaged on CBA plates containing 50 ng/ml ATc prior to transfer onto acidified urease plates. Change in colour due to urease activity is observed within 30 minutes after plate inoculation.

B. Strain hAD198 was grown on CBA plates in the absence of ATc and used to inoculate the whole urease plate. Discs containing 10 μl of 50 and 100 ng/ml ATc were placed on opposite ends of the plate. The *urePtetOV* promoter was induced in bacteria growing around the disc, and urease activity was observed within 20 h, and was more clearly evident after 40 h and 64 h.

C. As a negative control, urease negative mutant, X47 *ureA::rpsL-cat* (hAD176) was also plated onto urease plates. After 64 h of incubation, no colour change was observed.
Figure 6-11  ATc dose-dependent regulation of urease activity in *H. pylori* Tet-ON strains, hAD204-hAD208

Urease plate assay of Tet-ON strains transformed with *ptetR* (*P_{laA-tetR}* and different *urePtetO* constructs. Schematic diagram represents the position of the different *urePtetO* transformed strains. Fresh cultures of bacteria grown on CBA plates were collected and resuspended in PBS to OD$_{600}$ = 0.1. Aliquots of this suspension (20 μl) were spotted onto fresh urease plates containing ATc at concentrations ranging from 0 to 250 ng/ml. Plates were incubated under microaerobic conditions and examined for colour change every 24 h.
Figure 6-12  ATc dose-dependent regulation of urease activity in *H. pylori* Tet-ON strains, hAD214-hAD216
Figure 6-12  ATc dose-dependent regulation of urease activity in *H. pylori* Tet-ON strains, hAD214-hAD216 (adjacent page)

Urease plate assay of Tet-ON strains transformed with *ptetR6* (*P_{ihaT}-tetR*) and different *urePtetO* constructs. Schematic diagram represents the position of the different *urePtetO* transformed strains. Fresh cultures of bacteria grown on CBA plates were collected and resuspended in PBS to OD$_{600}$ = 0.1. Aliquots of this suspension (20 μl) were spotted onto fresh urease plates containing ATc at concentrations ranging from 0 to 250 ng/ml. Plates were incubated under microaerobic conditions and examined for colour change every 24 h.

The kinetics of *urePtetO* induction and repression was analysed in strain X47 *ptetR*4; *urePtetO*$_I$ (hAD204) and X47 *ptetR*4; *urePtetO*$_V$ (hAD208) by immunodetection of the UreB protein (Figure 6-13). After addition of 50 ng/ml ATc, UreB protein expression increased over time and reached maximum levels after 12 h and 8 h for *urePtetO*$_I$ and *urePtetO*$_V$ respectively (Figure 6-13 A). Withdrawal of ATc from induced cultures led to a significant decrease in UreB protein levels within 2 h demonstrating that both *urePtetO*$_I$ and V are quickly silenced (Figure 6-13 B) and that the UreB protein is turned over efficiently, dropping to the immunodetection threshold within 8 to 12 h.

### 6.4.3  Tet-regulation of *urePtetO* in vivo

Once it was established that *urePtetO* constructs could regulate the expression of urease in a tetracycline dependent manner *in vitro*, the next logical step was to test if tet-regulation could be used to regulate *H. pylori* urease phenotype *in vivo* and to study the effect a significant reduction in urease expression had on an established infections.

#### 6.4.3.1  Identification of *in vivo* tet-conditional X47 urease mutants

Experiments using wild-type X47 established that colonization could not be maintained in X47 challenged C57BL/6J mice when the animals were supplemented with more than 100 μg/ml Dox in their drinking water (section 4.4.4.2). To determine if *urePtetO* could be efficiently regulated *in vivo*, the Tet-ON conditional urease mutants were first tested for tetracycline dependent colonization. Tet-ON strains required tetracycline to induce urease expression and therefore to establish infection, which made it easier to identify the optimal tetracycline dose for *H. pylori* gene regulation *in vivo*. Tet-OFF conditional urease mutants will be tested in future studies using ATc to avoid tetracycline toxicity as higher concentrations of tetracyclines are required to repress urease expression in these strains.
Figure 6-13  Kinetics of urePtetO induction and repression

A. Time course of TetR-controlled induction of UreB expression. Bacteria were transformed as indicated and grown in HI media to mid-log phase (OD$_{600}$ = 0.5) before addition of 200 ng/ml of ATc. Aliquots of induced cultures were taken at indicated time points. *H. pylori* lysates were separated on a 10% SDS–PAGE gel. Equal amount of protein (~15 µg) was loaded into each lane.

Lanes 1 – 7, Time course of UreB induction by 200 ng/ml ATc
Lane 8, Control culture with no 200 ng/ml added

B. Time course of TetR-controlled repression of UreB expression. Bacteria were transformed as indicated and grown on CBA plates containing 200 ng/ml ATc for 48 h. Fresh bacteria were collected, washed with PBS and used to inoculate fresh HI media at a starting OD$_{600}$ = 0.5 and grown for 12 h. Aliquots of each culture were taken at indicated time points. *H. pylori* lysates were separated on a 10% SDS–PAGE gel. Equal amount of protein (~15 µg) was loaded into each lane.

Lanes 1 – 7, Time course of TetR repression of UreB expression
Lane 8, Control culture with 200 ng/ml ATc, maintaining UreB induction
In this study, a set of X47 Tet-ON conditional urease mutants was generated in which a range of different urease expression levels could be achieved in vitro by tet-regulation. Several Tet-ON strains with different induction properties were selected and tested for their ability to colonize C57BL/6J mice supplemented with different Dox concentrations that were within the tolerance of wild-type X47. Several experiments demonstrated that Dox supplementation could induce urePtetO sufficiently in vivo to facilitate colonization of conditional X47 urease mutants (Figure 6-14). Strains X47 mdaB::ptetR4; urePtetOI (hAD204) and X47 mdaB::ptetR6; urePtetOV (hAD216) could be re-isolated one week after oral challenge when animals were supplemented with 0.1 – 10 μg/ml and 5 - 20 μg/ml Dox respectively (Figure 6-14 A and B). Dox supplementation of 5 μg/ml was also sufficient to maintain strain X47 mdaB::ptetR4; urePtetOV (hAD208), which was successfully re-isolated two weeks after oral challenge (Figure 6-14 C). Strains with low levels or urease induction, X47 mdaB::ptetR4; urePtetOIV (hAD207) and X47 mdaB::ptetR6; urePtetOIV (hAD215), could not be re-isolated after oral challenge under the same experimental conditions. These experiments established that successful colonization of C57BL/6J mice could be achieved only with conditional urease mutants in which high levels of urease could be induced. Furthermore, their colonization was conditional, being dependent on low levels of Dox (1-20 μg/ml) supplementation.

6.4.3.2 Development of a conditional X47 urease mutant infection model

Strain X47 mdaB::ptetR4; urePtetOI (hAD204), was the most robust colonizer of the conditional urease mutants tested and also required less Dox supplement than the other strains. This strain was therefore used in further studies to test if urease was required by H. pylori once infection is established. Mice were challenged with pre-induced conditional urease mutant hAD204 and supplemented with Dox. Two weeks after oral challenge, when bacterial load in the stomach reaches a plateau (Ondek Pty. Ltd., unpublished data), Dox supplementation was removed and animals were sacrificed at several different time points. H. pylori infection was cleared between 3 and 7 days after Dox supplementation was withdrawn, however in the positive control group, only one out of six animals was still colonized after 4 weeks of Dox supplementation (Figure 6-15).
Figure 6-14  Infection of conditional urease mutants is dependent on Dox
Figure 6-14 Infection of conditional urease mutants is dependent on Dox
(adjacent page)

Urease expression in Tet-ON conditional urease mutant strains 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 Tet-ON strains and supplemented with a range of Dox concentrations in their drinking water. Wild-type infection data has been presented previously (Chapter 4, section 4.4.6.1). Bars represent mean bacterial load per group and points plotted represent colonization density for each individual animal. Detection limit was < 20 CFU per stomach (dotted horizontal line). Gastric specimens without *H. pylori* re-isolation are shown as null.

A. Bacterial load of mice (n = 3) one week after oral challenge with wild-type X47 (WT) or conditional urease mutant strain X47 *mdaB::ptetR4; urePtetOI* (hAD204). Dox concentration range tested was 0 – 1 μg/ml.

B. Bacterial load of mice (n = 3) one week after oral challenge with wild-type X47 (WT) or conditional urease mutant strain X47 *mdaB::ptetR4; urePtetOI* (hAD204) and X47 *mdaB::ptetR6; urePtetOV* (hAD216). Dox concentration range tested was 1 – 20 μg/ml.

C. Bacterial load of mice (n = 6) two weeks after oral challenge with wild-type X47 (WT) or conditional urease mutant strain X47 *mdaB::ptetR4; urePtetOV* (hAD208). Animals were supplemented with 5 μg/ml Dox.
Figure 6-15  Tet-regulation of urePtetO in vivo

Mice were orally challenged with pre-induced Tet-ON strain, X47 mdaB::ptetR4\textsuperscript{4}; urePtetOI (hAD204) and supplemented with 5 μg/ml Dox for two weeks. Dox supplement was withdrawn (red open squares) and animals were sacrificed 0, 1, 3, 7 and 14 days later. Control groups, animals that did not receive any Dox supplement and sacrificed on day 0 (open squares) and animals that were maintained on Dox for the duration of the entire experiment and sacrificed on day 14 (black squares). Horizontal bars represent median bacterial load per group (n = 6) and points plotted represent colonization density for each individual animal. Detection limit was < 20 CFU per stomach (dotted horizontal line). Gastric specimens without H. pylori re-isolation are shown as null.

This result warranted further optimization of the in vivo model for tet-regulated urease expression. During the construction of strain X47 mdaB::ptetR4\textsuperscript{4}; urePtetOI (hAD204) secondary mutations affecting colonization robustness may have occurred. Therefore clones of strain X47 mdaB::ptetR4\textsuperscript{4}; urePtetOI re-isolated from different animals, hAD222-7 (Table 3-2, hAD222-hAD227), were pooled together and evaluated for improved colonization. In addition, supplementation with the less toxic tetracycline analog, ATc, was also tested as a method for supporting increased bacterial load and improving the in vivo model. ATc supplementation improved infection rates in both animal groups challenged with the original lab strain hAD204 or the re-isolated conditional urease mutants hAD222-7 (Figure 6-16). Animal groups challenged with either hAD204 or hAD222-7, had similar infection rates. However, the bacterial load in mice supplemented with ATc and infected with strains hAD222-7 was significantly greater than in ATc supplemented animals infected with strain hAD204 and was also comparable to the bacterial load of ATc supplemented mice infected with wild-type X47. The experiment which tested if urease was required by H. pylori after infection has been established was repeated using these optimized conditions.
Mice groups were supplemented without (C) or with either 5 μg/ml of Dox or ATc in their drinking water. Animals were orally challenged with wild-type strain X47, with pre-induced Tet-ON strain, X47 mdaB::ptetR4; urePtetOI (hAD204) or a pool of pre-induced Tet-ON mouse re-isolates (hAD222-7). Animals were sacrificed two weeks after oral challenge. Bars represent mean bacterial load per group (n = 5) and points plotted represent colonization density for each individual animal. Detection limit was < 20 CFU per stomach (dotted horizontal line). Gastric specimens without \textit{H. pylori} re-isolation are shown as null.

Animals were infected with pre-induced strains hAD222-7 and supplemented with ATc for 2 weeks. Supplementation of ATc was ceased for half the animal groups and bacterial load was evaluated 0, 1, 3, 5 and 7 days later. On days 5 and 7, \textit{H. pylori} could still be re-isolated from mice supplemented with ATc but not from animals in which ATc supplementation had been withdrawn (Figure 6-17). This data confirmed that infection by conditional urease mutants required continuous tetracycline supplementation and demonstrated that urease is also required by \textit{H. pylori} to maintain a persistent infection. The combination of ATc supplementation and pooled conditional urease mutant re-isolates, hAD222-7, was used in all further experiments using the \textit{in vivo} model for tet-regulated urease expression.
Mice were orally challenged with pre-induced Tet-ON mouse re-isolate strains, hAD222-7, and supplemented with 5 μg/ml 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 < 20 CFU per stomach (dotted horizontal line). Gastric specimens without *H. pylori* re-isolation are shown as null.
6.4.4 Characterization of the host immune response upon urease restriction

The establishment of an in vivo model for conditional X47 urease mutants provided a system in which the role of urease in immune modulation and persistence could be studied in the context of an intact gastric environment. Preliminary studies using this model investigated if there were any significant changes in the host immune response upon down regulation of urease expression. Mice were challenged with wild-type X47 or pre-induced strains hAD222-7 and supplemented with ATc. Supplementation of ATc was withdrawn from half of the animal groups two weeks after oral challenge, and the animals were sacrificed at two early time points, framing the time where infection of the conditional strain is cleared, and one late time point.

6.4.4.1 Changes to bacterial population upon tet-repression of urease

Animals challenged with conditional X47 urease mutants, hAD222-7, and maintained continuously on ATc supplement had a consistent infection rate of 60% (Figure 6-18). Bacteria re-isolated from these groups were still conditional urease mutants, as tested in vitro after re-isolation, even after a total infection time of 8 weeks. Withdrawal of ATc supplement from the animal groups challenged with hAD222-7 resulted in reduced infection load on days 3 and 5 and, although not cleared, the infection rate had decreased to 20% on day 5. However, for the last hAD222-7 infected group, day 42, the bacterial load was higher and the infection rate had increased to 80%. H. pylori re-isolated from this last group of mice were all urease positive as, unlike the bacteria re-isolated at the early day 3 and day 5 time points, they were no longer conditional mutants as tested in vitro after re-isolation. Sequencing the ureA and mdaB loci of urease positive H. pylori clones re-isolated from each infected mouse identified mutations in urePtetO and the tetR gene (Table 6-1). Every mutation identified in the tetR sequence of these urease positive strains resulted in a non-conserved amino acid substitution in TetR, which likely introduced structural changes that inhibited homodimer formation. For some isolates however, no mutations were identified in these regions and so it is likely that compensatory mutations occurred in other chromosomal loci. Half of the animals in each group challenged with wild-type X47 were maintained on ATc supplement for the entire experiment. No differences in bacterial load or infection rate was observed between animals supplemented with ATc and those that
were not, demonstrating that long-term ATc supplement (5 μg/ml for 8 wks) does not interfere with *H. pylori* infection.

**Figure 6-18  *H. pylori* colonization upon urease restriction in vivo**

*H. pylori* colonization. Naive mice (n = 5) and mice challenged with wild-type X47 (WT) or pre-induced Tet-ON mouse re-isolate strains (hAD222-7) (n = 10) were supplemented with 5 μg/ml 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. Stomachs were homogenized in HI media after the lymphocyte isolation procedure. Serial dilutions of stomach homogenate were plated out to determine the number of CFU. Bars represent median bacterial load per group (n = 10) and points plotted represent colonization density for each individual animal. Detection limit was < 20 CFU per stomach (dotted horizontal line). Gastric specimens without *H. pylori* re-isolation are shown as null.
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Table 6-1  Mutations identified in the *mdaB* and *urePtetO* loci of urease positive *H. pylori* hAD222-7 clones re-isolated from infected animals

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mutations to TetR</th>
<th>Mutations to <em>urePtetO</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>A to C mutation in palindrome of upstream <em>tetO</em> site</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A to G mutation in -10 box</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A to G mutation after TSP</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>A to G mutation in -10 box</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A to G mutation after TSP</td>
</tr>
<tr>
<td>5</td>
<td>P167S</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>P167S</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Q116STOP</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Q116STOP</td>
<td>A to C mutation in palindrome of downstream <em>tetO</em> site</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A to G mutation in -10 box</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A to G mutation before TSP</td>
</tr>
<tr>
<td>9</td>
<td>G196W</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>G196W</td>
<td>-</td>
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<tr>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>A89V</td>
<td>A to C mutation in palindrome of upstream <em>tetO</em> site</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
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<tr>
<td>15</td>
<td>-</td>
<td>A to G mutation before and after TSP</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Refer to Figure 6-4 A for nucleotide sequence of *urePtetO*. |

6.4.4.2  Host antibody response

Antibodies against *H. pylori* whole cell lysate were measured in both the blood and the stomach. With the exception of one group, there was no correlation between the levels of X47 specific IgG and the colonization status of the animal. Overall, the antibody response against X47 in animals challenged with conditional urease mutants (hAD222-7) remained consistent through the course of the experiment. At earlier time points (less than three weeks after challenge) antibody titer in these groups was slightly higher than the animal groups which had been challenged with wild-type X47 (Figure 6-19 A). Interestingly, analysis of the IgG response measured in the same animals over an 8 week period demonstrated that IgG titers against X47 in animals infected with wild-type X47 increase significantly between 4 and 8 weeks of infection (the three animals that maintained low IgG titer were uninfected) (Figure 6-19 B). This same increase in IgG titer was not observed for the animals challenged with hAD222-7 as only slight increases in IgG titer were measured during the course of the experiment. IgA titers measured in the mouse stomach homogenate appeared to correlate with the IgG data, also suggesting that during early infection (< 3 wks) IgA response against
X47 may be greater in hAD222-7 challenged mice compared to mice challenged with wild-type X47 (Figure 6-19 C). Some of these differences do reach statistical significance (t-Test, P < 0.05) however these results must be confirmed using a greater number of animal stomachs which have not been processed for cellular infiltrate isolation for improved IgA detection.

**Figure 6-19** Host antibody response upon urease restriction *in vivo* (adjacent page)

Naive mice (n = 5) and mice challenged with wild-type X47 (WT) or pre-induced Tet-ON mouse re-isolate strains (hAD222-7) (n = 10) were supplemented with 5 μg/ml 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.

A. Anti-*H. pylori* IgG. The levels of IgG antibodies specific for *H. pylori* X47 antigen was measured for each animal at the time of sacrifice. Bars represent mean response per group (n = 5 or 10) and points plotted represent the response of each individual animal. Error bars represent standard deviation from the mean. No sera added (neg).

B. Anti-*H. pylori* IgG. Blood samples were collected at three different time points, 2, 4 and 8 weeks after oral challenge, from the animal groups sacrificed 42 days after ATc withdrawal. The levels of IgG antibodies specific for *H. pylori* X47 antigen was measured for each sample. Naive samples are aged matched controls. Bars represent mean response per group (n = 5 or 10) and points plotted represent the response of each individual animal. Error bars represent standard deviation from the mean. No sera added (neg). Statistical significance was evaluated using a Student’s t-Test * P < 0.05, ** P < 0.0025 for all animals in the group, ^ P < 0.0025 when data from uninfected animals were excluded.

C. Anti-*H. pylori* IgA. The levels of IgA antibodies specific for *H. pylori* X47 antigen was measured in the clarified stomach homogenate of each animal at the sacrifice time point. Bars represent mean response per group (n = 5 or 10) and points plotted represent the response of each individual animal. Error bars represent standard deviation from the mean No sera added (neg). Statistical significance was evaluated using a Student’s t-Test * P < 0.05.
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Figure 6-19 Host antibody response upon urease restriction in vivo

A
Anti-X47 IgG in sera

B
Anti-X47 IgG in sera - Timecourse

C
Anti-X47 IgA in stomach homogenate

Figure 6-19  Host antibody response upon urease restriction in vivo
6.4.4.3 Host cellular response

The host cellular response to *H. pylori* infection was measured in the gLN and at the site of infection, the stomach. The stained cell samples were first gated for live cells which were then segregated into several different lymphocyte populations. Representative data for the innate and adaptive immune cell population are presented: Neutrophils, the main cells involved in inflammation, Dendritic cells (DCs), involved in antigen sampling and presentation, and CD4+ cells, the main effector cells involved in *H. pylori* clearance (Aebischer et al., 2008; Mohammadi et al., 1997).

Technical difficulties were experienced with lymphocyte isolation from both gLN and stomach tissue, resulting in variable cell quality between samples. Data was of poor quality for many of the gLN samples due to the presence of fatty tissue which had not been efficiently removed. Overall, 1-7 million live cells were obtained for each gLN (Figure 6-20 A). There were no appreciable differences in the proportion of DCs (Figure 6-20 B), neutrophils (Figure 6-20 C) or CD4+ cells (Figure 6-20 D) in the live cell population isolated from the gLNs of different animal groups.

Strain X47 produces very little inflammation in the C57BL/6J mouse model and therefore very low numbers of immune cells were isolated from the stomach. Furthermore lymphocyte isolation was also variable due to slight differences in technique between experimenters. Significantly fewer cells were isolated from each stomach than from the gLNs, and therefore cells isolated from 5 stomachs had to be pooled to obtain $2.0 \times 10^4$ live cells on average per sample (Figure 6-21 A). From the limited data obtained, it appears that the proportion of DCs (Figure 6-21 B) and CD4+ cells (Figure 6-21 D) was reduced in the stomachs of *H. pylori* infected animals compared to naive animals. This reduction was greatest in animals infected with wild-type X47, followed by the conditional urease mutant hAD222-7 maintained on ATc. Loss of urease expression in hAD222-7, due to ATc withdrawal, seems to have resulted in DC and CD4+ cell proportions that were closer to that of naive animals on both day 3 and day 5. Interestingly for this treatment group, on day 42, when the stomach was populated by urease positive hAD222-7 bacteria, the DC and CD4+ cell proportions had decreased to levels that were between those of the hAD222-7 + ATc treated animals and the wild-type X47 infected animals. The proportion of neutrophils isolated from the mouse stomachs were slightly higher in *H. pylori* challenged animals and this difference was greatest on day 42 for mice infected with wild-type X47 and hAD222-7 +/- ATc.
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(Figure 6-21 C). At this last time point, the proportion of neutrophils in hAD222-7 + ATc treated mice was the lowest and similar to naive animals.
Figure 6-20 Host cellular response in gastric lymph node
Naive mice (n = 5) and mice challenged with wild-type X47 (WT) or pre-induced Tet-ON mouse re-isolate strains (hAD222-7) (n = 10) were supplemented with 5 μg/ml 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. The gLN were isolated and processed to form a single cell suspension. Cells were stained to identify different lymphocyte types within the live cell population. Bars represent mean per group (n = 5 or 10) and points plotted represent the value for each individual gLN. Error bars represent standard deviation from the mean.

A. Number of live cells isolated from each gLN

B. Proportion of live cells that were identified as dendritic cells

C. Proportion of live cells that were identified as neutrophils

D. Proportion of live cells that were identified as CD4⁺ cells
Figure 6-21  Host cellular response in the stomach
Naive mice (n = 5) and mice challenged with wild-type X47 (WT) or pre-induced Tet ON mouse re-isolate strains (hAD222-7) (n = 10) were supplemented with 5 μg/ml 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. Lymphocytes were isolated from each stomach and pooled, 5 stomachs per sample. Cells were stained to identify different lymphocyte types within the live cell population. Bars represent mean per group (n = 1 or 2) and points plotted represent the value for each individual stomach sample. Error bars represent standard deviation from the mean.

A. Number of live cells in each stomach sample

B. Proportion of live cells that were identified as dendritic cells

C. Proportion of live cells that were identified as neutrophils

D. Proportion of live cells that were identified as CD4+ cells
6.5 Discussion

The aim of this study was to generate a conditional *H. pylori* urease mutant, using the tet-system developed for *H. pylori* (Chapter 4), in order to study the role of urease in persistence and immune modulation *in vivo*. This study also generated additional tet-promoter architectures which permitted analysis of the relationship between the number and position of tetO sequences in tet-promoter regulation.

6.5.1 Tet-regulation of urePtetO in *H. pylori*

Conditional urease mutants of strain X47 were generated by placing the expression of ureA and ureB under the regulation of the Tn10-based tet repressor system adapted to *H. pylori*. Five different tet-promoter architectures based on the ureA promoter were generated. As observed in the regulation of uPtetO in GFP studies (Chapter 4), regulation or urePtetO was dependent on both the ptetR construct and on ATc concentration. In the Tet-OFF strains (revTetR expressing strains), urePtetO promoters could only be significantly regulated by the two strongest revTetR expressing constructs, ptetR5 and ptetR7. This suggests that the urePtetO promoters are stronger than the smaller uPtetO promoters as the weaker revTetR expression driven by PamiE in construct ptetR1, while sufficient to regulate GFP activity in uPtetO-GFP strains, was inefficient at regulating urease expression in urePtetO strains. The expression of TetR was sufficient to regulate urePtetO in all *H. pylori* Tet-ON strains, and as observed for uPtetO, the induction range and sensitivity to ATc was greatest in strains expressing the least amount of TetR protein, the strains transformed with ptetR4.

To understand how the location of tetO within PureA affected promoter activity, two promoter architectures, resembling well established tet-promoters used in *E. coli*, P_{LtetO-1} (Lutz and Bujard, 1997), (urePtetOI) and B. subtilis, P_{xyl/tet} (Geissendorfer and Hillen, 1990), (urePtetOII), were made in addition to the single, double and triple tetO promoter architectures used in Chapter 4 (urePtetOIII-V) (Figure 6-4). The urePtetO promoters varied in strength and, based on UreB expression, were all weaker than P_{ureA}. Interestingly, promoters urePtetOI, urePtetOII and urePtetOIV all had similar strengths and induction profiles, while, as observed for uPtetO2 in Chapter 4, urePtetOIV was a significantly weaker promoter compared to urePtetOV (equivalent to uPtetO1 in
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This last observation can be attributed to the decrease in AT content upstream of position -14, a moderately conserved feature in \textit{H. pylori} promoters (Sharma et al., 2010), due to \textit{tetO} sequence replacement. However, promoters \textit{urePtetO}I and \textit{urePtetOII} both have \textit{tetO} sequences in this region and have retained their strength compared to \textit{urePtetOV}. Therefore it is likely that both sequences upstream of the -10 position and downstream of the TSP are important to the basal strength of \textit{P}_{ureA}. Mutations to either one of these regions alone moderately decreases \textit{P}_{ureA} promoter strength by similar magnitudes, while mutations to both these regions of \textit{P}_{ureA}, \textit{urePtetOIII} and \textit{urePtetOIV} constructs, significantly decreases promoter strength.

Furthermore, based on published DNase I protection data (Pflock et al., 2005), introduction of \textit{tetO} sequences upstream of the -10 position most likely disrupted one of the ArsR–P operator sequences and therefore may have inhibited or decreased ArsRS mediated acid induction of urease expression. This potential disruption did not significantly affect colonization as all X47 \textit{urePtetO} (I-V) strains were still able to colonize the C57BL/6J mouse model and the conditional urease mutant, X47 \textit{mdaB::ptetR4; urePtetOI} (hAD204) was a more robust colonizer than X47 \textit{mdaB::ptetR4; urePtetOV} (hAD208), a strain whose ArsR–P operators remained intact.

To date, only the upstream ArsR–P binding region (in position -139 – -106) has been demonstrated, through mutagenesis, to be important in acid induction of urease (Pflock et al., 2005). It may be that the downstream ArsR–P binding region is not as critical for induction or that ArsRS mediated induction of urease expression is less important in the mouse model due to the higher gastric pH of mice compared to other animal models (Sachs et al., 2003). Future studies comparing acid induction of \textit{urePtetOI} and \textit{urePtetOII} with \textit{urePtetOV}, and identification of the ArsR–P operator sequences in \textit{P}_{ureA} by mutagenesis should prove insightful about the ArsRS regulatory system.

Comparison of the urease activity data with the total amount of UreB protein expressed by wild-type and \textit{urePtetO} strains revealed that under standard growth conditions a significant amount of urease is in the inactive apoenzyme form, which is in agreement with observations made in other studies (Maier et al., 2007; Stingl and De Reuse, 2005; van Vliet et al., 2002). \textit{urePtetO} regulation had a more significant effect on urease protein levels than on urease activity (Figure 6-22), suggesting that \textit{H. pylori} can sense how much of the total urease pool is in the active form and can ensure that a minimal
level of urease activity is maintained despite fluctuations in protein levels that are induced independently of the ArsRS and NikR regulatory systems.

![UreB protein and percent urease activity graph](attachment:urePtetOregulation.png)

**Figure 6-22  urePtetO regulation of urease activity and UreB protein levels**

This data was presented previously in Figure 6-5. Urease activity and UreB protein levels in X47 urePtetO(I-V) strains compared to wild-type X47. Fresh cultures of *H. pylori* grown on CBA plates were used to measure urease activity. Urease activity is expressed as a percentage of wild-type X47 (WT) urease activity. Bacteria from the same sample preparations were used to detect UreB protein. 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. Immunoblot data is representative of three independent experiments.

### 6.5.2 Conditional urease expression by *H. pylori* in vivo

The mouse infection studies using conditional X47 urease mutants demonstrated that tet-regulation of *H. pylori* virulence genes is possible *in vivo* and these studies successfully demonstrated that urease is required for persistent infection as established *H. pylori* populations were observed to crash within 5 days of TetR mediated restriction of urease expression.

Several experiments demonstrated that conditional urease mutants were able to colonize mice and that infection was dependent on tetracycline supplementation. The most robust conditional colonizer was strain hAD204 (mouse re-isolates hAD222-7) which had a strong urePtetOI promoter and was shown to be the most sensitive to ATc induction *in vitro*. Despite the decreased urease expression capabilities of strain hAD204 and other urePtetO strains, compared to wild-type, these strains were all able to establish infections, suggesting that *H. pylori* does not require the amount of urease it naturally
produces to colonize the mouse model, however the additional urease expression was certainly advantageous to the wild-type strain. The additional urease may allow the wild-type strain to neutralize acid or promote some other essential function (Eaton and Krakowka, 1994) more efficiently, and therefore replicate faster and reach the maximal bacterial burden while the *urePtetO* mutants struggle to efficiently replace bacteria shed from the gastric mucosa.

Several interesting observations can be drawn from the large animal experiment characterizing the bacterial and host responses upon down regulation of urease expression during infection. The first is that *H. pylori* conditional urease mutants could be maintained for 8 weeks without losing their conditional phenotype and that their *urePtetO* sequences remained intact after two passages through mice. This demonstrated that the *tet*-system in *H. pylori* is very stable in the absence of strong selective pressures and that it is suitable for studying gene function during persistent infection. Second, withdrawal of ATc supplement in animals infected with the conditional urease mutant resulted, as expected, in a steady drop in bacterial load and infection rate, and likely fell below the detection limit before day 7. However, later on day 42, this strain could once again be easily re-isolated although all the isolates constitutively expressed urease. This data confirms that urease is required by *H. pylori* to maintain the infection. Spontaneous mutations naturally arise in bacterial populations but, in the absence of strong selection, these mutants usually do not become dominant. It is likely that a small fraction of the *H. pylori* population accumulated spontaneous mutations during the first two weeks of infection that had either damaged the *tetO* sequence or had inactivated TetR so that urease expression could no longer be repressed. ATc withdrawal suppressed urease expression in the main *H. pylori* population, causing it to crash, and selected for the few mutants that were no longer *tet*-responsive, and six weeks was sufficient time for these mutants to multiply and re-populate the mouse stomach. The IgG data for this group of animals supports this theory as their IgG response against X47, unlike the group infected with wild-type X47, was still very low at 8 weeks despite the high bacterial load in their stomachs. This indicates that these animals were not exposed to a high bacterial load for a period of time that was long enough to stimulate an IgG response similar to that of the other two groups, which is consistent with a population crash during infection.
Chapter 6: Tet-regulation of *H. pylori* urease

There are two main limitations in the last *in vivo* experiment described, the infection rate of the X47 strains and the lack of inflammation induced by *H. pylori* in the mouse stomach. The incomplete infection by both Tet-ON and wild-type strains made it necessary to have large group sizes, which in turn limited the number of different animals groups. The lymphocyte isolation technique used in this study provided a means of quantifying the host cellular response in a way that cannot be achieved through histology. However, the low level of inflammation induced by strain X47 meant that very few lymphocytes could be isolated from each stomach, and therefore isolated lymphocyte populations had to be pooled together in order to obtain sufficient numbers for analysis by flow cytometry. This consequently reduced the number of samples for analysis and may have masked the true data as infiltrates from both infected and uninfected stomachs were combined. Furthermore, since lymphocyte isolation required processing of the whole stomach, very little additional analysis could be achieved for the site of infection. The bacterial burden could still be reliably measured however, the signal for IgA response was, unsurprisingly, significantly reduced in flushed stomachs compared to untreated tissue (Ondek Pty. Ltd., unpublished data). Despite these limitations, overall, the data suggest that there are differences in the host response between wild-type infected and conditional HP222-7 infected animals. Animals infected with urease positive bacteria seemed to have more inflammation and decreased numbers of DCs and CD4$^+$ cells in the stomach. The difference in number of DCs and CD4$^+$ cells may be due to increased activation and migration to the local lymph nodes, inhibition of their recruitment to the stomach or due to cell death. However, since the conditional urease mutant did not reach the same colonization density as the wild-type strain it is difficult to discriminate between the effect of antigen burden and the effect of urease levels on the host immune response.

Since the hosts cellular immune response is believed to be the most important component of the immune system for clearing *H. pylori* infection (Aebischer et al., 2008; Raghavan et al., 2010), experiments analysing cellular response should be pursued using an animal model that replicates the inflammation observed in human patients. The Mongolian gerbil is one example (Nakagawa et al., 2005; Wiedemann et al., 2009). Gerbils develop severe inflammatory cell infiltration after several weeks of *H. pylori* infection and they have much larger stomachs, meaning that there may be enough tissue to measure multiple infection parameters without having to compromise one or more assays. The major limitation to the Mongolian gerbil model however is the
limited number of antibodies that are available to study the gerbil immune system. The plethora of immunological tools is one significant advantage for the mouse model and the main reason it is so heavily used in *H. pylori* research. Transfer of the tet-system to a strain that does induce inflammation in the mouse model could be a good alternative to address the lack of inflammation induced by strain X47. However for future studies using either model, gerbil or mouse, the conditional *H. pylori* urease mutant must colonize with 90-100% efficiency to reduce the number of animals required in such studies.

It has been reported that neutralization of the gastric environment is not sufficient to permit colonization of urease negative mutant (Eaton and Krakowka, 1994). Urease is essential for acid resistance, and is likely also very important in nitrogen metabolism (De Reuse and Skouloubris, 2001) and immune modulation (Fan et al., 2000; Harris et al., 1996; Schoep et al., 2010). The tet-inducible system provides a genetic tool by which the different roles of urease in *H. pylori* infection can be teased apart to understand the mechanism for pathogen clearance upon knockdown of urease expression. Utilization of the tet-system overcomes the main obstacle to studying persistence and allows assessment of the role of urease and other proteins in pathogenesis once infection has been established. Future experiments using the tet-system could address the *in vivo* role of urease in nitrogen metabolism by testing if tet-suppression of urease expression during persistent infection can be rescued by amino acid supplementation (Williams, et al., 1996). The tet-system can also be used to construct a strain that expressed two urease genes, the native gene under tet-regulation and a second catalytically inactive urease that is constitutively expressed. Such a strain can be used to address the *in vivo* role of urease binding interactions with host cells. During infection, tet-regulation can suppress expression of the catalytically active urease while expression of inactive urease remains constitutive, maintaining urease binding interactions with epithelial and immune cells, and any changes in the kinetics of several infection parameters can be investigated.

Of additional interest would be to use the different urePtetO strains that lack TetR in long-term colonization experiments, as their range of urease expression is significantly lower than wild-type X47. The ability of urePtetO strains to colonize C57BL/6J mice demonstrates that, at least in mice, urease does not need to be expressed in such abundance by wild-type strains. It would be interesting to test if urePtetO strains can
colonize animals with more acidic stomachs, such as the gerbil, and if they do, to study what effects reduced urease expression has on bacterial fitness and on the host in terms of inflammation and disease progression.

One main observation drawn from this study is that the tet-system did not maintain long-term gene repression in *H. pylori* during infection. This is in contrast to similar studies for the *M. tuberculosis* animal infection model which has demonstrated the efficacy of such experiments investigating the long-term effects of tetracycline mediated gene regulation during persistent infection. This discrepancy reflects the differences in replication rate during persistent infection between the two pathogens. *M. tuberculosis* does not actively replicate during the persistence stage of infection and therefore there is a low incidence of mutation and little opportunity for selection and expansion of the mutant population. However this is not the case for *H. pylori*, which continually replicates during infection to replace the bacteria that are constantly being shed from the gastric mucosa and consequently there is sufficient opportunity for environmental pressures to act on the bacterial population and select for mutations that will overcome tet-regulation if those mutations provide a competitive advantage. The loss of the conditional urease phenotype in the long-term infection experiment does not mean that the tet-system, or any other conditional system, is not useful for the study of *H. pylori* infection *in vivo*. The result means that conditional gene expression systems can be used to answer specific questions, and that experiments have to be carefully designed within the limitations of such systems. Any experiment that places bacteria under pressure and provides them with sufficient time to adapt will always select for mutants that are better equipped to survive in the new environment\(^2\). The tet-system will work for experiments that investigate changes that occur during an early time window after a change in gene regulation is induced or for experiments in which altered gene expression does not put bacteria at a severe disadvantage, as with the knock down of essential urease.

One possible way to overcome the selection for mutations in the tet-system, so as to study the long-term effects of gene dysregulation, would be to adopt the Tet-ON

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\(^2\) Such experiments may prove very insightful regarding the ability of *H. pylori* to adapt to changing environments. With decreasing costs in genome sequencing and ever increasing computational power, it would be possible to study *H. pylori* adaptation by identifying the different genome wide compensatory mutations an *H. pylori* population may accumulate in order to counteract tetracycline induced regulation of important genes.
strategy developed for eukaryotes. In this strategy, the TetR protein must bind to the promoter in order to initiate transcription and maintain expression of the target gene, and promoter binding by TetR is induced by the presence of tetracycline. In such a system, withdrawal of tetracycline supplement will result in loss of gene function and any mutations in TetR or the tetO sequences will also result in a loss of gene function, making it more difficult for conditional mutants to remove tet-regulation by random mutation.

In conclusion, this study has generated and characterized a series of related tet-promoter architectures. Such a comparison has not been described in the literature. This study has allowed for a direct comparison between five different promoter architectures and characterized the relationship between the number of tetO sites, their position and their influence on the strength and regulation of the ureA promoter of H. pylori. This study has also demonstrated that the tet-system is stable in replicating bacteria in vivo and that it can be used to regulate H. pylori gene expression in the mouse model. Due to the urease mutant’s inability to colonize animal models, previously studies could only infer through indirect experiments that urease was required for persistence. The development of the tet-system for H. pylori has permitted direct testing and confirmation of this hypothesis. Using conditional urease mutants, this study has demonstrated directly that urease is required to establish and maintain infection even after H. pylori has reached the relatively neutral environment of the gastric mucosa. Future studies using conditional mutants will lead to the elucidation of the different functional roles of urease activity and its interactions with host cells in vivo.
7  

**dif recombination in *H. pylori***

7.1  **Introduction**

The faithful replication of chromosomal DNA and its appropriate distribution to daughter cells is one of the most important functions of the cell. During DNA replication, replication forks may become stalled or broken. There are several mechanisms by which replication forks can be healed and rebuilt, most of which rely on the action of recombination proteins and often results in homologous recombination events (Sherratt et al., 2004). In circular chromosomes, these homologous recombination events between sister chromosomes can generate a chromosome dimer which must be resolved into separate monomers and segregated to daughter cells before cell division (Sherratt et al., 1995). The resolution of dimeric chromosomes is catalysed by the Xer site-specific recombination system. In *E. coli*, the mechanism of site specific recombination to resolve chromosomal dimers has been extensively studied (Barre and Sherratt, 2005). Dimer resolution requires the action of XerC and XerD site-specific recombinases at a 28-bp recombination site known as *dif* which is located within the chromosome terminus region. XerC and XerD act as heterodimers, with each Xer protein having a distinct role in *dif* recombination (Arciszewska et al., 2000). FtsK and topoisomerase IV (TopoIV) are two additional key enzymes that are involved in the coordinated resolution and segregation of newly replicated chromosomes. FtsK is a multifunctional protein localized in the septum (the site of cell division) where its C-terminal domain is involved in ATP-dependent DNA translocation to position and synapse the two *dif* sites as well as to activate dimer resolution by XerCD (Aussel et al., 2002). TopoIV is a type II topoisomerase. It is an essential enzyme (Kato et al., 1990) that plays a vital role in chromosome replication by decatenating the thousands of topological links between interlocked daughter chromosomes formed during replication, and works in coordination with FtsK and XerCD (Figure 7-1) (Grainge et al., 2007).
Figure 7-1  Model of chromosome replication, decatenation and segregation
Chapter 7  dif recombination in H. pylori

Figure 7-1  Model of chromosome replication, decatenation and segregation
(adjacent page)

Single line represents double stranded DNA (except of top centre and top right diagrams), green circle represents oriC and yellow triangle represents dif, newly synthesized DNA is represented in blue. During DNA replication, replication forks may become stalled or broken and are healed by recombination between growing sister chromosomes. TopoIV removes initial pre-catenation (interlinks between newly replicated sister chromosomes) formed during replication, allowing the chromosomes to start segregation. When DNA replication is complete, FtsK segregates newly replicated chromosomes into the daughter cells and the action of TopoIV continues to remove the interlinks between sister chromosomes. If no exchanges or an even number of exchanges between sister chromosomes occurred during replication, the product of replication will be two catenated monomers which can be unlinked by TopoIV. If an odd number of exchanges occurred, completion of replication will produce a single circular knotted dimer. TopoIV activity can untie the knot but the molecule will remain a circular dimer unless recombination occurs. FtsK synapses the two sister dif sites and activates dimer resolution by XerC and XerD which proceeds through a holiday junction intermediate. Once the chromosomes are resolved into monomers, cell division can be finalized. (Adapted from (Grainge et al., 2007; Kuempel et al., 1991))

dif/Xer recombination systems have been detected computationally in many phyla (Carnoy et al., 2002; Kono et al., 2011) including Proteobacteria (Carnoy and Roten, 2009), Firmicutes (Le Bourgeois et al., 2007; Sciochetti et al., 2001) and Archae (Cortez et al., 2010; Duggin et al., 2011). In silico analyses of proteobacterial genomes revealed that more than 85% contain a conventional E. coli dif/XerCD type system. However, a subgroup of ε-proteobacteria, including Helicobacter spp. and Campylobacter spp., was inferred to contain an atypical dif/Xer system, consisting of just a single Xer recombinase, which was named XerH (Figure 7-2) (Carnoy and Roten, 2009). Single Xer recombinase systems have been reported in the gram-positive Streptococci and Lactococci (XerS) (Le Bourgeois et al., 2007) and in Archaea (XerA) (Cortez et al., 2010; Duggin et al., 2011) but have not been confirmed in proteobacteria. ftsK homologues are found in nearly all eubacterial species including the ε-proteobacteria and Streptococci and Lactococci, but interestingly, several slow growing bacterial pathogenic genera, including Helicobacter, Campylobacter and Mycobacterium, lack TopoIV subunit genes, parC and parE (Ambur et al., 2009). In the case of H. pylori, inference about XerH action is complicated further due to the presence of a second divergent xer recombinase gene, xerT, usually located within a large TnPZ transposon found in many strains (Fischer et al., 2010; Kersulyte et al., 2009). Although its encoded XerT protein is needed for transposon excision and conjugative transfer, XerT may also collaborate with XerH for chromosome resolution (as with XerCD in E. coli).
Figure 7-2  Phylogenetic tree of Xer recombinases

XerH from subgroup of ε-proteobacteria, represented mostly by Helicobacter and Campylobacter species, compared to XerC and XerD recombinases, from representative α, β, δ and γ taxa and other ε species, and to XerS recombinases, from Firmicutes. There is no phylogenic association between the single recombinases, XerS and XerH. (Figure taken from (Carnoy and Roten, 2009))

Therefore to improve our understanding about Helicobacter and Campylobacter biology, which often behave so differently from the bacterial model organism, E. coli, the in silico identification of XerH and dif in the ε-proteobacteria subgroup was characterized genetically using H. pylori as a representative organism. The study entitled ‘Xer recombinase and genome integrity in Helicobacter pylori, a pathogen without topoisomerase IV’ was published in PLoS One (attached in section 7-2). The experiments presented demonstrate site-specific recombination at H. pylori difH sites, and show that it requires XerH, FtsK and an intact difH sequence, but not XerT. The study also brings into focus the need to elucidate how catenanes are processed in the many other slow growing pathogens that, like H. pylori, lack TopoIV. Additionally, arising from the characterization of the difH/XerH system was the adaptation of a new genetic tool for generating markerless H. pylori mutants.
A novel and simple technique utilizing \textit{dif} sequences and XerCD recombinases was recently reported for chromosomal gene replacement and subsequent excision of antibiotic resistance markers in \textit{B. subtilis} and \textit{E. coli} (Bloor and Cranenburgh, 2006). By using endogenous bacterial Xer recombinases this technique, termed “Xer-cise”, circumvented the requirement for exogenous site-specific recombinase systems and thereby simplified genetic manipulations for obtaining markerless mutants.

Generating a markerless mutation in \textit{H. pylori} usually requires two transformation steps (section 2.2.3.3) and the final mutant can be obtained in 2-3 weeks. Confirmation of the \textit{H. pylori} Xer machinery and \textit{difH} sequence meant that Xer-cise could be adapted for genetic manipulation in this and other members of the \textit{difH}/XerH harbouring \varepsilon\text{-}proteobacteria. Applying Xer-cise in combination with \textit{rpsL-cat} counterselection permitted the generation of markerless mutants in \textit{H. pylori} within 8-10 days and was demonstrated to work in \textit{H. pylori} strains 26695, G27 and X47. This work is described in the manuscript entitled ‘\textbf{Xer-cise in Helicobacter pylori: one step transformation for the construction of markerless gene deletions}’ (attached in section 7-3), and the Xer-cise technique is now routinely used in the Marshall laboratory.

The intensive study of \textit{E. coli} as a model organism has provided significant insights into how living systems address fundamental challenges, such as energy acquisition, replication and survival under environmental stress. Many mechanisms elucidated in \textit{E. coli} serve as good models for other bacterial species, however variations in these mechanisms have evolved, especially in more distantly related genera. Identifying and understanding the differences between the molecular mechanisms characterized in \textit{E. coli} and those identified in other bacteria will help facilitate the adaptation of the genetic tools first established in \textit{E. coli} research to the study of other bacteria. Characterization of the \textit{difH}/XerH system in \textit{H. pylori} is one such example, where discovering the differences between the model and the organism of interest has facilitated the adaptation of a new genetic tool that will expedite the generation of mutants in an organism in which its leisurely growth rate significantly increases experimental times and consequently delays research outcomes.
7.2 Paper: ‘Xer recombinase and genome integrity in *Helicobacter pylori*, a pathogen without topoisomerase IV’

Experiments in this paper were conceived and designed by: Aleksandra W. Debowski, Christophe Carnoy, Alma Fulurija, Douglas E. Berg, Barry J. Marshall and Mohammed Benghezal.

The experiments described in this paper were performed by: Aleksandra W. Debowski, Christophe Carnoy, Phebe Verbrugghe, Hans-Olof Nilsson, Jonathan C. Gauntlett, Tania Camilleri and Mohammed Benghezal.

The data was analyzed by: Aleksandra W. Debowski, Christophe Carnoy, Phebe Verbrugghe, Hans-Olof Nilsson, Douglas E. Berg, Barry J. Marshall and Mohammed Benghezal.

The manuscript was written by: Aleksandra W. Debowski, Christophe Carnoy, Douglas E. Berg and Mohammed Benghezal.

Overall contribution of Aleksandra W. Debowski to this paper was 65%.
Xer Recombinase and Genome Integrity in Helicobacter pylori, a Pathogen without Topoisomerase IV

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Abstract

In the model organism E. coli, recombination mediated by the related XerC and XerD recombinases complexed with the FtsK translocase at specialized dif sites, resolves dimeric chromosomes into free monomers to allow efficient chromosome segregation at cell division. Computational genome analysis of Helicobacter pylori, a slow growing gastric pathogen, identified just one chromosomal xer gene (xerH) and its cognate dif site (difH). Here we show that recombination between directly repeated difH sites requires XerH, FtsK but not XerE, the TnPZ transposon associated recombinase. xerH inactivation was not lethal, but resulted in increased DNA per cell, suggesting defective chromosome segregation. The xerH mutant also failed to colonize mice, and was more susceptible to UV and ciprofloxacin, which induce DNA breakage, and thereby recombination and chromosome dimer formation. xerH inactivation and overexpression each led to a DNA segregation defect, suggesting a role for Xer recombination in regulation of replication. In addition to chromosome dimer resolution and based on the absence of genes for topoisomerase IV (parC, parE) in H. pylori, we speculate that XerH may contribute to chromosome decatenation, although possible involvement of H. pylori’s DNA gyrase and topoisomerase III homologue are also considered. Further analyses of this system should contribute to general understanding of and possibly therapy development for H. pylori, which causes peptic ulcers and gastric cancer; for the closely related, diarrheagenic Campylobacter species; and for unrelated slow growing pathogens that lack topoisomerase IV, such as Mycobacterium tuberculosis.


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Introduction

Crossovers between circular monomeric chromosomes generate dimers and interlocked (catenated) structures that cannot segregate properly at cell division [1]. Bacteria with circular chromosomes generally contain site-specific tyrosine Xer recombinases that act at cognate dif sites near where replication terminates and that resolve chromosome dimers to free monomers [1,2]. Deletion of dif or inactivation of a xer recombinase gene causes formation of abnormally partitioned nucleoids and cell filamentation in E. coli type model organisms [3]. Cell filamentation results from SulA-mediated inhibition of cell division, is induced in the SOS response to DNA and chromosome breakage [4]. Chromosome dimer resolution in most bacterial species, including E. coli, is mediated by two related recombinases, XerC and XerD, that function as a pair of heterodimers and that target a 28-bp dif site. The dif site is presented to the Xer complex by the FtsK DNA translocase protein. FtsK is anchored at incipient cell division septa, and interacts with a set of oriented and highly repeated 8 bp named KOPS sequences (FtsK-orienting polar sequences). The E. coli chromosome’s dif region is rich in KOPS sites, which are in opposite orientation on each side of the dif site. Orientation-specific KOPS recognition and asymmetry in the KOPS distribution direct FtsK-chromosome interactions to effectively guide presentation of dif to the XerC-XerD complex for recombination. FtsK interacts specifically with the XerD component of the XerCD complex and with several other proteins including Topoisomerase IV, which are likely to be important for efficient, well-regulated chromosome separation and segregation [2,5,6]. Many thousands of topological links arise as circular chromosomal DNAs are unwound during replication [7]. In E. coli interlocked (catenated) chromosomes are resolved efficiently to...
monomers by topoisomerase IV [8], which is essential [9], primarily because of its high capacity to resolve interlocked chromosomes, and thereby allow efficient chromosome segregation, apace with rapid cell division [8]. Of note, DNA gyrase (responsible for DNA supercoiling) and Xer recombination may play secondary roles in decatenation [7,8,10]. This is illustrated by the ability of E. coli’s XerCD-df FsK system to substitute for topoisomerase IV [11] to remove catenane links between circular DNA molecules in vitro without topoisomerase IV [11]; and the suppression of a temperature sensitive (conditional lethal) topoisomerase IV mutation by XerCD-df recombination in E. coli producing an engineered FsK protein with no septum anchor (FsK50C) that is thus soluble in the cytoplasm [7]. This Xer-mediated resolution of catenated DNAs entails multiple interconversions of catenated monomers and knotted dimers, removing a link at each step.

Xer/dif recombination systems have been detected computationally in many phyla [12,13] including Proteobacteria [14], Firmicutes [15,16] and Archaea [17,18]. Our in situ analyses revealed that more than 85% proteobacterial species contain a conventional E. coli-type system in which XerC and XerD recombinases act as heterodimers on cognate dif sites, with each Xer protein having a distinct role. However, Helicobacter pylori, the gastric pathogen implicated in peptic ulcer disease and gastric cancer, was inferred to contain just a single Xer recombinase, which was named XerH, as do the related Campylobacters, which cause diarrheal disease, and all other members of Helicobacter pylori’s epsilon proteobacteria and Streptococci [15] and in Archaea (XerA) [14]. Single Xer recombinase systems were also found in the Gram positive Streptococci and Lactococci (XerS) [15] and in Archaea (XerA) [14,17,18], FsK homologues are found in nearly all subcellular species including the epsilon proteobacteria and Streptococci and Lactococci. Interestingly, several slow growing bacterial pathogenic genera, including Helicobacter, Campylobacter and Mycobacterium, lack parC and parE topoisomerase IV subunits genes [19].

Further complicating inferences about XerH action in the case of H. pylori, many strains contain a second divergent xer recombinase gene, xerT, generally within a large Tn916 transposon [20,21]. Although its encoded XerT protein is needed for transposon excision and conjunctive transfer, and probably also functions as a transposase, the possibility of XerT collaborating with XerH for chromosome resolution (as with XerC and XerD in E. coli) also merited testing.

The experiments presented here demonstrate site-specific recombination at H. pylori difH sites, and show that it requires XerH, FsK and an intact difH sequence, but not XerT, and bring into focus the need to learn how catenanes are processed in the many other slow growing human pathogens that, like H. pylori, lack topoisomerase IV.

Materials and Methods

Bacterial strains and culture conditions

The H. pylori strains and plasmids used in this study are listed in Table S1. Streptomyacin resistant rpsL-mutant strains were used for transformation with the difH repeat (rpsL-cat containing) cassette [22,23]. H. pylori strains were routinely 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 in H. pylori was carried out by supplementing media with chloramphenicol, streptomycin, and/or kanamycin at final concentrations of 10 μg/ml Escherichia coli DH5α was grown in Luria-Bertani broth. When necessary, antibiotics were added to the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 20 μg/ml. H. pylori cultures were incubated at 37°C in sealed jars using the Anaerobert™ MarkII system (Mart Microbiology B.V., The Netherlands) after one atmosphere replacement using the following gas composition N2:H2:CO2, 85:5:10.

Oligonucleotides.

The oligonucleotides used in this study are listed in Table S2.

Assays of difH site recombination

To test if the putative 46-bp H. pylori dif sequence (difH) ATTTAAAAGTTGAAATGTCGAGTTTTCCATACCTAAATGA was functional, a recombination assay was developed using a cassette containing both selectable (cat) and counterselectable (rpsL) genes, rpsL-cat (streptomyacin susceptibility, chloramphenicol resistance, respectively), flanked by difH sites [22,24,25]. This cassette was generated by PCR amplification from genomic DNA containing rpsL-cat using primers DifHPF and DifHPR, which contain direct repeats of difH and BamHI restriction sites near their ends. A control cassette containing 40 bp of sequence unrelated to difH was generated with primers NondifL and NondifR. These PCR products were cloned as described [26] to create plasmids pHint_difH-RCAT-difH and pHint_nondif-RCAT-nondif. Constructs were sequenced using primers SEQidF and SEQidR to ensure that difH and ‘nondif’ sequences were intact. Natural transformation of a derivative of H. pylori strain 26695 made resistant to streptomyacin by mutation in its normal rpsL gene (called 26695m) with pHint_difH-RCAT-difH and pHint_nondif-RCAT-nondiff was used to place these cassettes in the H. pylori chromosome between genes HP0203 and HP0204 (strains 26695m \( H. pylori \) HP0203 and 26695m \( H. pylori \) HP0204–nondif– RCAT, respectively). Chromosomal DNAs from the resulting chloramphenicol-resistant transformants were checked for streptomyacin sensitivity (which is dominant to resistance in rpsLm and rpsLm WT partial diploids). These transformants and their descendants were also checked for correct insertion of the difH repeat cassette and for recombination at difH sites by PCR using primers 2003F and 2004R. Additionally, chloramphenicol-resistant transformants were subcultured on streptomyacin-containing agar when needed to select for or to quantify rates of loss of the cassette. Chromosomal DNAs of representative streptomyacin-resistant, chloramphenicol-sensitive derivatives were sequenced to confirm recombination at difH sites as diagrammed in Figure 1.

Due to the low difH recombination rates in the HP0203-HP0204 intergenic region, the difH repeat cassette was also placed at the ureABC locus for further studies. Synthetic sequences containing direct repeats of wild-type or mutant difH sites separated by a BglII restriction site and flanked with BamHI sites were synthesized and cloned into plasmid pMA-RQ from Genecart (AG Regensburg, Germany) (mutant difH sequences shown in Table S1). A BamHI fragment containing the rpsL-cat cassette was cloned into BglII digested plasmids pDifWT, pDifM1, pDifM2, and pDifM3 (Table S1) to give rpsL-cat flanked with WT or mutated difH sequences in pDifWT-RC, pDifM1-RC, pDifM2-RC, pDifM3-RC respectively (Table S1). These difH flanked DNAs were excised with BamHI and cloned into plasmid pUreAB [27], a Bluescript-derived plasmid containing regions of homology for chromosomal replacement of [or insertion between] the ureAB and ureC (urease) genes. This resulted in plasmids pUreAB_DifWT-RC, pUreAB_DifM1-RC, pUreAB_DifM2-RC and pUreAB_DifM3-RC, which were used for transformation of H. pylori strain 26695m to create 26695m uraC_pDifWT-RC, uraC_pDifM1-RC, uraC_pDifM2-RC and uraC_pDifM3-RC. Using primers UreABF and UreaBR, chromosomal DNA of the
resulting chloramphenicol-resistant transformants was checked for the correct insertion of the difH repeat cassette and for difH recombination at the ureAB locus.

H. pylori mutants used to assess H. pylori xerH and xerT roles in difH recombination

xerH is the only xer recombinase gene found in every H. pylori genome, although many strains, including 26695 (used here), also contain another xer family gene, xerT [21]. Strain 26695<sup>sm</sup> derivatives with null mutations in these xer genes were constructed in order to test each gene's role in dif site recombination. xerH::rpsL-cat and xerT::rpsL-cat insertion mutant constructs were generated by PCR with overlapping primers [28,29]. Briefly, for xerH (HP0675), DNAs flanking and including much of this gene were amplified from 26695 genomic DNA using primers XerHrcat1 and XerHrcat2, and XerHrcat3 and XerHrcat4, respectively; and the rpsL-cat cassette was amplified using primers XerHrcat5 and XerHrcat6. Nested primers XerHrcat7 and XerHrcat8 were used for splicing overlap extension (SOE) PCR to generate a DNA segment containing rpsL-cat inserted within the HP0675 ORF, which would be suitable for transformation. The same strategy, using primers XerTrcat1 through XerTrcat8, was used to generate xerT::rpsL-cat mutant of the xerT gene (HP0995). Natural transformation of H. pylori strain 26695<sup>sm</sup> with these products yielded 26695<sup>sm</sup> xerH::rpsL-cat and 26695<sup>sm</sup> xerT::rpsL-cat.
DNAs of transformants were checked for correct allelic insertion by PCR. Simple unmarked xerH and xerT deletion alleles (ΔxerH and ΔxerT) were then made by SOE PCR. Briefly, for xerH, 1-kb DNA segments flanking HP0675 were PCR amplified using primers XerHdel1 and XerHdel2, and XerHdel3 and XerHdel4, and fused in a second SOE PCR using nested primers XerHdel5 and XerHdel6. The ΔxerT allele was made similarly with primers XerTdel1 through XerTdel6. H. pylori strains containing xerH::rpsL-cat and xerT::rpsL-cat constructs were transformed with corresponding simple deletion DNAs, streptomycin resistance was selected, and transformants were screened for loss of chloramphenicol resistance and further checked by PCR. This resulted in strains 26695ΔΔxerH and 26695ΔΔxerT.

To assess the role of XerH and XerT in δH recombination, these ΔxerH and ΔxerT strains were transformed with pHInt_difH-RCAT-diiH to give 26695ΔΔxerH, HP0675::F-diiH-RCAT and 26695ΔΔxerT, HP0675::F-diiH-RCAT; or with pUreAB_DvWT-RC to give 26695ΔΔxerH, ureB::difHWT-RC and 26695ΔΔxerT; ureB::difHWT-RC (δH Banking rpsL-cat at the ureB locus). Chloramphenicol-resistant transformants were selected, and then assayed for δH site recombination by appearance of streptomyacin resistance and loss of chloramphenicol resistance and by PCR.

Complementation of xerH mutation

The xerH (HP0675) ORF was amplified from 26695 genomic DNA using primers XerHf and XerHRr and the product was cloned downstream of the strong ureA promoter of pTrpA-up (Table S1) to create pTrpA-xerH construct. Transformation of strain 26695ΔΔxerH with pTrpA-xerH yielded 26695ΔΔxerH/ureAcomp, which was in turn transformed with pTrpA-upXerH to create strain 26695ΔΔxerH comp, ΔxerH complemented with highly expressed xerH gene at the chromosomal xerH locus. XerH mutant strains complemented at the xerH locus by ureA promoter control. Chromosomal DNAs of the resulting transformants were checked by PCR for correct allelic replacement at xerH and to verify that the cloned xerH gene was not at the normal chromosomal xerH locus. Two independently generated 26695ΔΔxerH comp clones were then transformed with pUreAB_DvWT-RC to give 26695ΔΔxerH comp; ureB::difHWT-RC (δH Banking rpsL-cat at the ureB locus). Chloramphenicol-resistant transformants were selected and assayed for recombination events at δH sites.

Construction of H. pylori mutants used to assess susceptibility to DNA damage

SOE PCR was used to generate a rpsL-cat::difH-lac construct. For rpsL-cat (HP0877), 1-kb DNA segments flanking HP0877 were amplified from 26695 genomic DNA using primers RuvCrecat1 and RuvCrecat2, and RuvCrecat3 and RuvCrecat4 respectively; and the rpsL-cat cassette was amplified using primers RuvCrecat5 and RuvCrecat6. Nucleotide primers RuvCcreat7 and RuvCcreat8 were used to generate a rpsL-cat::difH-lac containing SOE PCR product. Natural transformation of strain 26695 with pTrpA-xerH under conditions as described for 26695 was performed to confirm the xerH derivatives of X47 (which already contains an rpsLstreptomyacin resistance allele), this strain was transformed with genomic DNA from strain 26695, xerH::rpsL-cat. Genomic DNA isolated from the resulting transformants was used as template for PCR to confirm rpsL-cat insertion at xerH. A pool of X47 xerH:rpsL-cat clones was transformed with the PCR product obtained from 26695 ΔxerH (unmarked deletion), with selection for streptomyacin resistance and chloramphenicol susceptibility.

Several attempts to construct the DNA segment to make a recA knockout by SOE PCR failed. Therefore, in vitro transposition was used to insert the rpsL-cat cassette into recA. The HP0153 ORF was amplified by PCR, using primers RecAF and RecAR, and cloned into pGEMT-Easy (Promega, Madison, WI) to create pRecA. In vitro transposition of a segment containing rpsL-cat flanked by ends of phage Mu into pRecA was done using MuA transposase (Finnzymes, Finland, F-750) to generate a library of pRecA-RC clones. H. pylori strain 26695ΔΔxerH was transformed to chloramphenicol resistance using this plasmid library to generate 26695ΔΔxerH::mu-rpsL-cat. Chromosomal DNA was isolated from transformants, and insertion of the mu-rpsL-cat-tnu cassette into ORF HP0153 was confirmed by PCR. A similar strategy was used to truncate the H. pylori ftsK homologue. Primers FtsKF and FtsKR were used to amplify the HP1090 ORF and generate the ftsK containing plasmid pFtsK. In vitro transposition of mu-rpsL-cat into pFtsK generated a library of pFtsK-RC mutant DNAs which was used in transformation to make strain 26695 ftsK::mu-rpsL-cat. Transformants were characterised by PCR and DNA sequencing, and one with an insertion at the 454th codon from ftsK’s 3′ end was identified.

Double and triple mutants were obtained by transforming 26695ΔΔxerH with PCR products containing recC::rpsL-cat to create 26695ΔΔxerH::recC::rpsL-cat (Table S1). Genomic DNA isolated from the resulting transformants was used as template for PCR to confirm rpsL-cat insertion at xerH. A pool of X47 xerH::rpsL-cat clones was transformed with the PCR product obtained from 26695 ΔxerH (unmarked deletion), with selection for streptomyacin resistance and chloramphenicol susceptibility. Genomic DNA from the resulting X47 ΔxerH transformants was used as template for PCR to confirm clean deletion of xerH, as described for 26695.

To generate the recA mutant in X47 background, wild-type X47 was transformed with genomic DNA from strain 26695 ΔxerH::rpsL-cat. Genomic DNA isolated from the resulting transformants was used as template for PCR to confirm rpsL-cat insertion at the recA locus as described for 26695.

UV susceptibility assay

Fresh cultures of H. pylori, passaged the day before, were suspended in PBS (pH 7.2) and standardized to an OD600 = 2. 50 μl aliquots of bacterial suspension was placed into a single well of a UV transparent 96 well plate (NUC) and exposed to UV light at 312 nm using the TFX-35M transiluminator (LifeTechnologies, Carlsbad, CA) at a distance of 43 cm for 0 to 75 sec. Serial dilutions of irradiated cells were plated onto CBA plates and incubated at 37°C for four days. Colonies were counted and percent survival was calculated. UV susceptibility experiments were repeated at least three times on two independent clones for each H. pylori strain.
DNA content analysis by Fluorescence Activated Cell Sorting (FACS)

The DNA content analysis was performed as described [31]. Briefly, _H. pylori_ cells were grown on blood agar plates and inoculated in BHI medium containing 10% Newborn Calf Serum (NCS) at OD₆₀₀ = 2 and incubated in 100 µl in 96 well plate overnight (18 h) with shaking at 100 rpm. The bacteria were collected by centrifugation, the pellet was resuspended in 300 µl PBS and added to 900 µl PBS with 4% paraformaldehyde and samples were incubated 15 min at room temperature. The bacterial pellet was washed and resuspended in PBS. The analysis was performed with a Becton Dickinson FACSCanto II Flow cytometer.

Oxidative stress susceptibility assay

Susceptibility to oxidative stress using 2 mM and 20 mM paraquat was tested on Columbia agar plate using the disc method as described [32].

Antibiotic Sensitivity Testing

Wild-type, ΔxerH mutant and xerH complemented strains were inoculated on Columbia agar plates. A single E-test strip (AB Manufacturer's Instructions) was placed in the centre of each plate and the plates were incubated at 37°C for 6 days. The minimal inhibitory concentration (MIC) for ciprofloxacin was determined according to the manufacturer’s instructions.

Growth curves

Fresh cultures, passaged the day before, were resuspended in BHI. 5 ml of BHI containing 10% NCS and Dent (Oxoid) was inoculated with 100 µl of a stock inoculum standardized to OD₆₀₀ = 2. Growth studies were performed without any prior adaptation of _H. pylori_ strains to liquid media. Growth was measured every 4 to 12 h for up to 40 h. Each experiment was done in duplicate and repeated at least twice.

Electron microscopy

_H. pylori_ grown on Columbia agar were collected and washed in PBS (pH 7.4), prefixed in 2.5% glutaraldehyde in PBS buffer for 1 h, and then rinsed in PBS. After post-fixation in 1% osmium tetroxide (in PBS), samples were dehydrated through ascending gradient of ethanol and then critical-point dried using carbon dioxide. Samples were sputter coated with palladium (4 nm) and examined using a SEM (Zeiss 1555 VP SEM) at 3 KV and a working distance of 6 mm.

Phylogeny of the single Xer recombinases

Phylogenetic analysis of the Xer recombinase proteins was performed with MEGA version 4 [33]. Sequences were aligned with ClustalW, and the phylogeny was built using the Neighbor-Joining method [34].

Experimental Infection of Mice

_P. helicobacter_ free C57BL/6J mice were purchased from the Animal Resource Centre (Perth, Western Australia). Studies were performed with approval from the UWA Animal Ethics Committee (approval no. 07/100/598). Each eight-week-old mouse was orogastrically inoculated with approximately 10⁹ CFUs of _H. pylori_ harvested from an overnight agar plate culture into BHI broth. Colonisation of mice inoculated with X47 wild-type or its ΔxerH mutant was evaluated 2 weeks after challenge as described [29].

Results

The _difH_ sequence undergoes site-specific recombination in _H. pylori_

An excision assay in _H. pylori_ strain 26695 was designed to mimic chromosome dimer resolution (Figure 1A) and to test the ability of the recently identified _difH_ site [14] to undergo site-specific recombination. This assay used a DNA segment with direct repeats of _difH_ flanking _ypsL_ and _cat_ genes, which confer susceptibility to streptomycin and resistance to chloramphenicol, respectively. In our first tests this “_difH_ repeat” cassette was inserted in the _H. pylori_ chromosome between genes HP0203 and HP0204, which is about 525 kb from the normal _difH_ site (Figure S1). A negative control strain contained an equivalent cassette, but with 40-bp of other DNA (‘nondif’) in place of _difH_ at this same chromosomal location. In PCR tests, each of two bacterial clones containing the _difH_ repeat cassette yielded two DNA fragments: 2.1 kb, expected of recombination between _difH_ sites; and 3.6 kb, the full-length cassette (not recombinant at _difH_ sites) (Figure 1B), whereas clones containing the _nondif_ repeat cassette yielded only the 3.6 kb PCR product (Figure 1B). This outcome indicates that _difH_ sites placed at a new chromosomal locus can undergo site-specific recombination.

_difH_ recombination at the HP0203-HP0204 locus was also characterised by restoration of streptomycin resistance, which results from loss of the _ypsL_ (susceptible) allele ( _difH_ recombination), or from rare gene conversion between the added _ypsL_ gene in this cassette and the resistant allele at the normal chromosomal _ypsL_ locus [22]. Under our conditions, no (<0.1%) streptomycin resistant colonies were obtained when the 40 bp yeast DNA (‘nondif’) sequence flanked the _ypsL-cat_ cassette. In contrast, many streptomycin resistant colonies were obtained in strains in which _difH_ flanked _ypsL-cat_. All of these streptomycin resistant colonies were chloramphenicol sensitive. PCR confirmed that streptomycin sensitive clones still contained the full-length _difH_ repeat cassette and that _ypsL-cat_ was absent from streptomycin resistant clones. As expected, DNA sequencing confirmed that a single _difH_ “scar” sequence had been retained in these streptomycin resistant, chloramphenicol sensitive excisants, (Figure 1 and data not shown). The frequency of XerH recombination at _difH_ sites at the _ureAB_ locus (about 647 kb from _difH_ site; Figure S1) was also evaluated using this _difH_ repeat cassette. Cells were grown for 2 and 4 days in non-selective medium (no chloramphenicol), streaked out for single colony isolates, and 100–200 colonies were then tested for streptomycin/chloramphenicol resistance/susceptibility phenotypes. Figure 1C shows that the _difH_ recombination frequency is significantly higher for the cassette placed at _ureAB_ than at HP0203-HP0204 after 4 days culture. We conclude that the chromosomal position of paired _difH_ sites affects the frequency of recombination between them.

_difH_ site recombination requires the single XerH recombinase

_xerH_ was the only xer recombinase gene identified computationally in every fully sequenced genome of the Campylobacterales order, which includes the genus _Helicobacter_ [14]. However, many _H. pylori_ strains also contain another xer-like recombinase gene named _xerT_ (named for its association with the Tn7Z transposon) [20,21]. In frame deletions were made in both _xerH_ and _xerT_ to test each of them for possible roles in _difH_ site-specific recombination. Using the _difH_ repeat cassette at the _ureAB_ locus and our PCR
Xer Recombination and Genome Integrity

assay, we found that xerH deletion blocked difH recombination, whereas xerT deletion did not (Figure 2A). In addition, no excision was detected in the ΔxerH mutant by testing ~200 colonies for the emergence of streptomycin resistant, chloramphenicol sensitive colony phenotypes after 2 and 4 days growth on non-selective medium, whereas the isogenic ΔxerT mutant strain has undergone as much recombination as the xer-wild-type strain, or possibly more, after 2 and 4 days incubation (Figure 2B). Complementation of the ΔxerH allele with an intact xerH gene under a strong urease promoter (where it is probably over-expressed) restored difH site-specific recombination (Figure 2C). Indeed, the high level of the small PCR product in XerH complemented strains (difH recombination product; >80% of total) suggested that the amount of XerH protein per cell may be tightly controlled, and that increased XerH protein markedly increased the excision frequency, at least for difH at ectopic sites. The plasmid used to introduce the difH repeat cassette into the H. pylori chromosome (pHInt_difH-RCAT-difH) did not undergo difH site recombination in E. coli (data not shown), indicating that E. coli’s own XerC and XerD recombinases do not process difH; this was as expected, given difH and E. coli dif sequence divergence (Table 1 and [14]). However, expression of XerH in E. coli promoted excision at difH sites in 10% of this plasmid population (estimate based on plasmid restriction enzyme digest profile; data not shown). Taken together, our results indicate that recombination between difH sites is mediated by just one Xer recombinase without other species-specific factors and that XerH may be limiting, at least for difH sites far removed from their normal location. They further suggested that XerT might inhibit XerH, since more difH recombination was seen in the ΔxerT strain than in its wild-type parent (Figure 2B), although further testing is needed to learn if the stimulation seen in a ΔxerT strain reaches statistical and thus biological significance.

Point mutations in difH sequences that block XerH-mediated recombination

Before performing a mutational analysis of the difH, the nucleotide frequency at each position was calculated over 50 bp from 24 epsilon proteobacterial species to establish a consensus sequence (Table 2, Table S3). This revealed that difH consists of two highly conserved regions (position 17 to 23 and 29 to 34) separated by a variable region (position 24 to 26 and 28) and two other highly conserved positions, 10 and 14 (Figure 3A). In addition, positions 17 to 22 and 29 to 34 are always in a palindrome (inverted repeat), whereas positions 13, 23, 28, and 38 are not (Figure 3B). Thus, the difH sequence can be viewed as two matched domains flanking a unique 6 bp central region.

difH point mutations were made and tested for their ability to undergo difH recombination in H. pylori when at the ureAB locus. PCR tests showed that replacement of G by C in palindrome position 18 or of the four As by four Ts in palindrome positions 19 to 22 each abolished difH recombination in each of two independent clones (Figure 4A, lanes difH M1 and difH M2). difH recombination also seemed to be reduced by a C to G mutation at the non-palindromic but highly conserved position 23, although this mutation’s effect seemed leaky (very weak excisant PCR product; Figure 4A, lane difH M3). In confirmation, tests for the appearance of streptomycin resistant clones showed that the difH M2 mutation abolished difH recombination, and that the difH M3 mutation caused a severe impairment of recombination (1±1% for difH M3 vs. 9±2% for difH-wild-type after 4 days incubation on non-selective medium; Figure 4B).
FtsK is required for XerH recombination

The presence of an ftsK homologue (HP1090) in H. pylori suggested that XerH action might require the FtsK DNA translocase, much as does XerC/D action in E. coli. To test the role of H. pylori’s putative FtsK translocase protein, we deleted the 3’ terminal 1212 bp of the 2580 bp ftsK gene to generate a strain whose FtsK protein is truncated, and missing the γ regulatory domain that in the E. coli mediates FtsK interaction specifically with XerD, and not with the related XerC recombinase, for XerC/XerD mediated dif recombination [2]. Our mutant protein retains the N-terminal membrane anchor domain, which is essential for viability [35]. No difH recombination was detected by PCR in an H. pylori strain containing our mutant ftsK gene and the difH repeat cassette at the ureAB locus (Figure 5). We conclude that XerH mediated recombination between difH sites depends on FtsK in H. pylori, even for difH sites far from the normal difH locus (Figure S1), and despite H. pylori’s use of a single Xer-type recombinase.

Table 1. Consensus difH sequence obtained from complete H. pylori genomes.

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(1) difH sequences were retrieved from H. pylori complete genome sequences downloaded from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).
## Table 2. Consensus dif\(_h\) sequence obtained from 24 epsilon proteobacteria species.

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</table>

**CONSENSUS**(3): TTTTATTTATTTATGATGAAAACTAAATTGTAATTTTCAAGTAAAAATTAATAAAA

1) Uncompleted genome sequences were noticed as “uncompl.”
2) Nucleotides in bold characters are common to all 24 dif\(_h\) sequences.
3) If the nucleotide frequency represents more than 50%, the nucleotide is written in upper case letters; otherwise, the nucleotide is written in lower case letters.

doi:10.1371/journal.pone.0033310.t002

Xer Recombination and Genome Integrity
In *E. coli* FtsK guides chromosomal translocation and presentation of *dif* to the Xer complex by a set of asymmetric KOPS sequences (5'-GGGNAGGG) whose distribution is skewed toward *dif* and the replication terminus and polarized (most on leading DNA strand during bidirectional chromosome replication). Although this octamer is also abundant in *H. pylori* genomes, we think it is unlikely to serve as KOPS for *H. pylori*’s FtsK protein because its genomic distribution is neither skewed near *difH* nor highly polarized. The only *H. pylori* octamer mimicking *E. coli*’s KOPS in its distribution is 5'-AGTAGGGG-3' (Figure S1). This octamer was identified earlier by Hendrickson and Lawrence in their survey of many bacterial genomes [6] as a putative chromosome “architecture imparting sequence” for *H. pylori*. In accord with their view, we propose that it serves as *H. pylori* FtsK’s guide for *difH* presentation, its KOPS sequence.

Slight growth defect, UV and ciprofloxacin susceptibility, and resistance to oxidative stress of *DxerH* mutant

Light and scanning electron microscopy indicated that loss of *xerH* did not cause filamentation in *H. pylori* equivalent to that caused by loss of *xerC* or *xerD* in *E. coli* (Figure 6A and 6B). The Δ*xerH* mutant grew less well than its wild-type parent did (Figure 6C), as did a derivative of this Δ*xerH* strain complemented by a highly expressed intact *xerH* gene (Figure 6C). These outcomes suggest that *XerH* levels are regulated – that either too much or too little can be deleterious.

Studies in *E. coli* showing that *xerC* inactivation exacerbated the moderate UV susceptibility that is caused by a *ruv*-deficiency [36] prompted us to test if *xerH* inactivation affects *H. pylori*’s UV susceptibility. Figure 6D shows that *xerH* inactivation caused UV sensitization, albeit less extreme than that caused by *recA* or *ruvC* inactivation, and that normal UV resistance was restored to a *DxerH* mutant by complementation with a functional *xerH* gene. Inactivation of *xerH* in a *DruvC* mutant did not affect this strain’s normally very high UV sensitivity, whereas enhanced UV sensitivity was observed in a *DxerG* mutant with increased recombination [37] compared to either mutant alone (Figure 6D). The Δ*xecA ΔxerG* or Δ*xuc* Δ*xerG* double and Δ*xerH Δxuc* Δ*xerG* triple deletion strains had the same phenotype as Δ*xecA* or Δ*xuc* single deletion strains (data not shown). Two failed attempts to obtain a Δ*xecA* derivative of a Δ*xerH* strain prevented assessment of UV sensitivity in the absence of both *xerH* and

![Figure 3. *difH* sequences.](image-url)
RecA-mediated generalized recombination. Deletion of sequences encoding the FtsK gamma (probable XerH interaction) domain caused moderate UV sensitization, almost as much as that caused by ΔxerH itself (Figure 6E). Ciprofloxacin induces DNA double-strand breaks that are repaired by RecA- and RuvABC-mediated homologous recombination [38]. The ΔxerH mutant was more sensitive to ciprofloxacin than wild-type (Table 3) despite H. pylori's functional recA and ruvABC genes. This suggested a function other than DNA repair for XerH (e.g., dimeric and catenated chromosome resolution). The complemented ΔxerH mutant (overexpressing XerH) was more resistant than the ΔxerH mutant, again indicating a function other than DNA repair. Finally, the oxidative stress resistances of ΔxerH and ΔftsK mutant H. pylori were similar to that of wild-type, as were ΔrecA, ΔrecG and ΔrecG mutant strains (Figure S2). This outcome indicates that XerH recombination is not needed for base pair excision repair in H. pylori. Since the generalized recombination that UV, recG deletion and ciprofloxacin promote should result in formation of dimeric and catenated chromosomes, we suggest that failure to resolve such topological structures underlies the ΔxerH and ΔftsK mutant phenotypes. We propose that Xer recombination resolve chromosome dimers in H. pylori and speculate a role of XerH/difH in chromosome decatenation.

Impaired chromosome segregation in ΔxerH mutant

As noted above, an inability to resolve chromosome dimers and catenated chromosomes should block chromosome segregation at cell division. The lack in H. pylori of homologues of parC and parE, which in E. coli encode the two subunits topoisomerase IV, suggested that XerH-mediated recombination might also be used for chromosome decatenation. To test this idea, the DNA contents of wild-type, ΔxerH mutant and complemented ΔxerH mutant strains were analysed by flow cytometry after staining of DNA with Hoechst dye, much as in other studies of the hokA chromosome replication initiation gene [39]. This showed that ΔxerH mutant cells contained more DNA on average than their wild-type parents (Figure 7). In order to specifically test that XerH could perform decatenation, uncomplicated by chromosome dimers, which arise by generalized (RecA-mediated) recombination, we would have needed a ΔrecA ΔxerH double mutant. However, as noted above, we were unable to construct this double deletion strain.

We also note that the DNA contents of XerH complemented cells, which have increased XerH activity, was higher than those of isogenic wild-type cells (Figure 7). This suggests that excess XerH protein stimulates initiation of chromosome replication, or conceivably, that it interferes with chromosome segregation (reminiscent of that seen when XerH protein is absent). Taken together, these results suggest that ΔxerH mutants do not undergo efficient chromosome segregation, that they accumulate subpop-
ulations, one with multiple (dimeric and perhaps entangled) chromosomes, and one without chromosomal DNA. A chromosome segregation defect is in line with a failure to resolve dimeric and catenated chromosomes, structures that Xer recombination can resolve in *E. coli* [7].

**XerH is needed for gastric colonisation**

The ΔxerH mutant’s apparent defect in chromosome segregation and lack of severe growth phenotype in *vitro* prompted us to test if xerH is needed by *H. pylori* in its gastric mucosal environment. The ΔxerH allele, and for comparison, a ΔnuC allele, were transformed into strain X47, which colonizes mice robustly. C57BL/6J mice were inoculated orogastrically with mixtures of these mutant strains and their isogenic X47 wild-type parent; the mice were sacrificed two weeks later and gastric mucosal levels of *H. pylori* were assayed by bacterial culture. Compared to the robust stomach colonization observed for wild-type *H. pylori* (WT), the ΔxerH mutant did not colonize mice at all (Figure 8). Interestingly, the ΔnuC mutant, which exhibits a more severe DNA repair defect, did colonise mice, although with a 5-fold lower bacterial load than wild-type (Figure 8). The inability of the ΔxerH mutant to survive in the gastric niche contrasts with ΔnuC mutant colonization, and further supports the idea that XerH is not involved in DNA repair, but rather in chromosome maintenance such as chromosome dimer resolution and possibly in chromosome unlinking. This, in turn, suggests that the slow growing *H. pylori* depends on a unique chromosome replication and maintenance machinery to thrive in its special gastric niche.

### Discussion

The present study confirmed our computational prediction [14] that *H. pylori* uses just one dedicated tyrosine recombinase, XerH, for site-specific recombination at a cognate chromosomal *dif* site (*difH*) – not a pair of distinct proteins akin to XerC and XerD tyrosine recombinases of *E. coli* and most other eubacterial species. For our experiments, we constructed a cassette with direct repeats of *difH* sites flanking counterselectable and selectable genes (*rpsL* and *cat*, respectively) and placed this “*difH* repeat” cassette at

---

**Table 3. Ciprofloxacin susceptibility of *H. pylori* strains.**

<table>
<thead>
<tr>
<th>Strains</th>
<th>MIC (μg/ml)</th>
<th>Median</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>ΔxerH</td>
<td>0.079</td>
<td>0.079</td>
</tr>
<tr>
<td>xerH complemented</td>
<td>0.250</td>
<td>0.250</td>
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</table>

1Minimum inhibitory concentration.  
295% confidence interval based on the Wilcoxon signed rank test of eight independent experiments.  
3doi:10.1371/journal.pone.0033310.t003
arbitrarily chosen \textit{H. pylori} chromosomal locations. \textit{DifH} recombination was detected by loss of the \textit{pdml-cat} segment, as scored by bacterial phenotype or PCR. Deletion of \textit{xerH} blocked recombination between \textit{difH} sites in the \textit{H. pylori} chromosome; and conversely, \textit{xerH} expression promoted recombination between them in a plasmid in \textit{E. coli}. The related \textit{XerT} recombinase, present in many but not all \textit{H. pylori} strains [14], was not needed for \textit{difH} recombination. This fits with \textit{XerT}'s usually being associated with a widespread conjugative transposon, but not a fixed component of every \textit{H. pylori} genome [20,21]. Single \textit{Xer} proteins are also used for recombination at cognate \textit{dif} sites in \textit{Lactococcus} [15], \textit{Streptococci} [15] and related Gram positive genera [16], and in \textit{Archaea} [17,18], but they are distinctly phylogenetically from \textit{H. pylori}'s \textit{XerH} and \textit{XerT} (Figure 9).

Our \textit{\Delta xerH} mutant \textit{H. pylori} exhibited a general DNA segregation defect. No typical filamentation was observed during normal growth (Figure 6), as in \textit{E. coli \textit{xerC} or \textit{xerD} on \textit{dif} site mutant strains} [49], or under UV stress (data not shown). This discrepancy may be explained by \textit{H. pylori}'s lack of an SOS response and \textit{E. coli}-type cell division inhibitor (SulA), which is induced by the DNA breakage [4] that occurs when cell division proceeds without chromosome dimer resolution. Alternatively, the lack of filamentation in \textit{H. pylori \Delta xerH} mutant might be ascribed to \textit{H. pylori}'s much longer doubling time (3–4 hours) and small genome size (one-third \textit{E. coli}'s). Although \textit{E. coli} chromosome replication takes some 40 min, rapidly growing \textit{E. coli} (20 min generation time) can undergo multiknot replication. In consequence, rounds of replication can initiate in one cell cycle, finish in the next cell cycle, and still allow segregation in which each daughter cell receives at least one complete genome. There should be no such need for multiknot replication in \textit{H. pylori}, with its small genome size, and leisurely growth rate. We propose that these features underlie the lack of filamentation in \textit{\Delta xerH} mutant \textit{H. pylori}.

Deletion of \textit{xerH} in \textit{H. pylori} caused: (i) a slight growth defect in liquid culture (Figure 6C), as is typical of \textit{\Delta xer} mutants of \textit{E. coli} [40] (ii) a markedly increased sensitivity to DNA breakage inducing and homologous recombination stimulating UV irradiation (Figure 6, D and E), and ciprofloxacin (Table 3), (iii) an increased UV sensitivity of a \textit{xerG} mutant [37], (iv) an increased cellular DNA content (Figure 7), which we interpret as a defect in chromosome segregation; and (v) an inability to colonize mice (Figure 8). Overexpression of \textit{xerH} in our complementation experiments also increased the level per cell. This unexpected finding suggests a role of XerH in regulation of DNA replication/ segregation and merits further study. We also found that an intact FtsK DNA translocase protein was needed for \textit{difH} recombination (Figure 5), presumably for effective \textit{difH} site presentation to \textit{H. pylori}'s \textit{XerH} recombination complex at the end of each DNA replication cycle, and much as expected based on \textit{E. coli} results [2,16,17,41,42,43,44]. Xer recombination likely depends on and is regulated by cognate FtsK proteins in all eubacterial species, although, curiously not in \textit{Archaea}, since they lack obvious \textit{ftsK} genes [2,16,17,41,42,43,44]. Deletion of \textit{H. pylori} FtsK's cytoplasmic domain, including its putative \gamma segment, which probably interacts with XerH, led to a loss of \textit{difH} site recombination. This implies FtsK-XerH interaction for coordinated control of chromosome dimer resolution and segregation to daughters at cell division. Our finding of \textit{XerH}-dependent \textit{difH} recombination in \textit{E. coli} raises the possibility of low level FtsK-independent recombination in \textit{H. pylori} as in Vibrios [43] or lack of FtsK-Xer interaction specificity as observed in \textit{Streptococci} [41].

The importance of the wild-type \textit{difH} sequence was confirmed by finding that each of our several \textit{difH} sites mutations interfered with \textit{difH} recombination (Figure 4). \textit{difH}'s short inverted repeats, which flank a small central unique sequence spacer, have a slight asymmetry (e.g., left arm positions 10 and 14) that is well conserved among \textit{epidus} proteobacteria (Figure 3A). We speculate that this asymmetry could be used to determine the time and place of the FtsK-XerH complex's DNA cleavages: first on one \textit{difH} strand, and then the other – reminiscent of the sequential cleavages by the phylogenetically distinct single XerS recombinase [41], and formally equivalent to the different roles and timing of action of \textit{E. coli}'s \textit{XerC} and \textit{XerD} proteins in its heterodimeric recombinase [45]. Our computational analysis further identified the sequence 5'-AGTAGGGG, whose polarized clustering near \textit{difH} make it a prime candidate for \textit{H. pylori}'s KOPS, this octamer had also been noted earlier, and was formally proposed as a chromosome “architecture imparting sequence”, but without suggesting a molecular terms [6]. \textit{H. pylori}'s putative KOPS diverges markedly from \textit{E. coli}'s KOPS octamer (Figure S1), a feature that should encourage comparison of FtsK-KOPS binding and associated interactions in these two species.
The reason for our inability to delete recA in a ΔxerH mutant strain is unclear, but might suggest a second important role for difH recombination. For example, many thousands of topological links created by unwinding and replication of double stranded circular DNAs must all be removed for proper chromosome segregation at cell division. The great majority of such links are removed in E. coli by topoisomerase (Topo) IV, which interacts with and is stimulated by FtsK. However, H. pylori seems to lack this essential enzyme: it has no close homologues of the Topo IV encoding parC and parE genes [19], and thus must use some other enzyme system. The possibility that XerH/difH recombination could allow H. pylori to avoid a chromosome decatenation dilemma is suggested by findings of E. coli XerC/D and dif-dependent (although inefficient) DNA decatenation in vitro; and by the in vivo XerC- and XerD-dependent suppression of a temperature sensitive (conditional lethal) Topo IV mutation when mutant soluble form of FtsK is overproduced [7]. We think that the case for XerH-mediated decatenation in H. pylori would be strengthened if it were shown that this suppression reflects fulfillment of Topo IV’s functions by a more effective XerC/ XerD/FtsK complex, not just FtsK-mediated stabilization of an impaired (temperature sensitive) Topo IV [11]. It is interesting in this context, that obvious parC and parE (Topo IV) homologues are found in Lactococci and Streptococci, which also use just a single Xer recombinase, these Gram positive species do not challenge our conventional understanding of how chromosomes are decatenated [19] in the way that H. pylori does. As a third case, Archaea, use just a single Xer recombinase (XerA) but lack obvious homologues of parC and parE and FtsK [17]. Assuming that they will have developed yet another solution to the decatenation problem, valuable insights should emerge from detailed comparisons of daughter chromosome separation and chromosome integrity maintenance in diverse microbial species. One possible solution emerges from finding of higher decatenase activity in the DNA gyrase of M. tuberculosis and M. smegmatis than of E. coli [46,47].

Although we can speculate that H. pylori chromosome decatenation is mediated by iterated round of XerH action on difH, we can also imagine DNA gyrase-mediated decatenation in H. pylori. This would be in accord with our ΔxerH strain’s increased susceptibility to the gyrase inhibitor ciprofloxacin (Table 3), and E. coli DNA gyrase’s low efficiency decatenation of linked circular DNAs in vitro (superimposed on its very efficient DNA negative supercoiling). As a final alternative, we can also imagine H. pylori’s topoisomerase III (HP0116) mediating sufficient decatenation, by extrapolation from Topo III’s activity in E. coli [48].

The analyses presented here suggest many valuable experiments for future studies, bringing into focus the need to learn how catenanes are processed in the many other slow growing human pathogens that, like H. pylori, lack topoisomerase IV. Particularly informative should be further molecular genetic and enzymologic analyses of H. pylori’s XerH, DNA gyrase and TopoIII, in the context of this pathogen’s small genome size and leisurely growth rate. The lessons learned should be applicable to the understanding, diagnosis and therapy for diverse pathogens and conditions: H. pylori itself, and peptic ulcer disease and gastric cancer; the closely related Campylobacters and associated diarrheal diseases; and equally, unrelated pathogens such as Mycobacterium tuberculosis, which chronically infects many millions of people worldwide, also without obvious genes for Topoisomerase IV.
Supporting Information

Figure S1  Positions of putative KOPS sequences and other features in the H. pylori 26695 genome sequence. The H. pylori 26695 genome sequence was scanned for the octamer AGTAGGGG sequences that had been implicated computationally as likely to affect chromosome architecture [6] (A), and for the GGNNAGGG octamer that constitutes the KOPS sequence of E. coli [49] (B). The circular H. pylori genome is presented here as a linear structure, with ends corresponding to its origin of bidirectional replication. The AGTAGGGG and GGNNAGGG octamers are represented by red and blue plain diamonds, respectively. Also indicated are the locations of ureA and difH, and the HP0203-HP0204 and ureA loci at which we had placed difH repeat cassette.

(TIF)

Figure S2  Sensitivity of H. pylori mutants to oxidative stress. Sensitivity to oxidative stress was evaluated in a disk assay using 2 mM or 20 mM of paraquat on blood agar plates that had previously been streaked for confluent growth with either mutant or wild-type cells as indicated. Following a 3–4 day incubation period, the clear zones surrounding the disks were measured. The experiments were repeated three times and standard deviation is indicated.

(TIF)

References


3. Carnoy C, Roten CA (2009) The features in the 26695 genome sequence were scanned for the octameric AGTAGGGG repeat cassette.


7.3 Manuscript: ‘Xer-cise in *Helicobacter pylori*: one-step transformation for the construction of markerless gene deletions’

Experiments in this manuscript were conceived and designed by: Aleksandra W. Debowski, Jonathan C. Gauntlett, Hong Li, Tingting Liao, Miriam Sehnal, Hans-Olof Nilsson, Barry J. Marshall and Mohammed Benghezal.

The experiments described in this paper were performed by: Aleksandra W. Debowski, Jonathan C. Gauntlett, Hong Li, Tingting Liao, Miriam Sehnal and Hans-Olof Nilsson.

The data was analyzed by: Aleksandra W. Debowski, Jonathan C. Gauntlett, Hong Li, Tingting Liao, Miriam Sehnal, Hans-Olof Nilsson, Barry J. Marshall and Mohammed Benghezal.

The manuscript was written by: Aleksandra W. Debowski and Mohammed Benghezal.

Overall contribution of Aleksandra W. Debowski to this manuscript was 70%.
‘Xer-cise in Helicobacter pylori: one-step transformation for the construction of markerless gene deletions’

Running title: one-step gene deletion

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Abstract

Xer-cise is an efficient selectable marker removal technique that was first applied in *Bacillus subtilis* and *Escherichia coli* for the construction of markerless gene deletions. Xer-cise marker excision takes advantage of the presence of site-specific Xer recombination in most bacterial species for the resolution of chromosome dimers at the *dif* site during replication. The identification and functional characterization of the *difH*/XerH recombination system enabled the development of Xer-cise in *Helicobacter pylori*. Markerless deletions were obtained by a single natural transformation step of the Xer-cise cassette containing *rpsL* and *cat* genes, for streptomycin susceptibility and chloramphenicol resistance respectively, flanked by *difH* sites and neighboring homologous sequences of the target gene. Insertion/deletion recombinant *H. pylori* were first selected on chloramphenicol-containing medium followed by selection on streptomycin-containing medium for clones that underwent XerH mediated excision of the *rpsL-cat* cassette, resulting in a markerless deletion. This strategy eliminates the need for multiple passages on non selective plates and subsequent screening of clones for loss of the antibiotic cassette by replica plating. XerH-mediated removal of the antibiotic marker was successfully applied in three different *H. pylori* strains to obtain markerless gene deletions at very high efficiencies. An unmarked triple deletion mutant was also constructed by sequential deletion of *ureA*, *vacA* and HP0366 and removal of the selectable marker at each step. The triple mutant had no growth defect suggesting that multiple *difH* sites per chromosome can be tolerated without affecting bacterial fitness.
Introduction

The natural transformation and homologous recombination characteristics of *H. pylori* have facilitated genetic studies of its pathogenesis, involving mutagenesis and subsequent characterization of the corresponding mutants’ phenotype both *in vitro* and *in vivo* [1,2,3,4,5,6,7]. Insertion mutagenesis is a common bacterial genetic method, involving the insertion of a selectable marker to disrupt or replace the gene of interest. Studies entailing multiple mutations within the same strain, the comparison of several alleles in one genetic background or interference with either neighbor gene expression or cellular physiology, would all benefit from the removal or recycling of the selectable marker to generate unlabeled mutations. In *H. pylori*, removal of the selectable marker to produce markerless mutations has been achieved by using counterselectable cassettes based on the *sacB* gene encoding for the levansucrase, conferring toxicity in presence of sucrose [8], the *rpsL* wild-type allele, conferring streptomycin sensitivity to streptomycin resistant recipient strains harboring a mutated *rpsL* allele [9], or the *rdxA* gene, conferring metronidazole susceptibility to metronidazole resistant strains harboring a *rdxA* deletion [10]. Although counterselection is a powerful genetic tool for the precise introduction, replacement or deletion of DNA sequences in the genome of *H. pylori*, a second transformation step is required to replace the counterselection cassette, doubling the time required to obtain markerless mutants.

Here we developed a one-step transformation mutagenesis strategy in *H. pylori* based on Xer-mediated excision of the selectable marker as described by Bloor et al. [11]. In a previous report, the single recombinase *difH*/XerH system was confirmed experimentally in *H. pylori* [12]. The *difH* sequence was shown to promote site-specific recombination that was dependent on XerH
only. This result confirmed the presence of a single recombinase dif/Xer system in *H. pylori* for the resolution of chromosome dimers during bacterial replication. Taking advantage of XerH recombination in *H. pylori* and combining it with the counterselection cassette based on *rpsL*, a one-step transformation technique was developed to obtain markerless deletions. XerH-mediated removal of the antibiotic marker, previously named Xer-cise [11], was successfully applied to obtain markerless gene deletions in three different *H. pylori* strains. An unmarked triple deletion mutant was also constructed by sequential deletion of *ureA*, *vacA* and HP0366, followed by the removal of the selectable marker at each step, highlighting the usefulness of Xer-cise in the construction of *H. pylori* mutants.

**Methods**

**Bacterial strains and culture conditions**

The plasmids and *H. pylori* strains used in this study are listed in Table 1. The primers used in this study are listed in Table 2. *H. pylori* strains were routinely grown at 37°C under microaerobic conditions on Columbia blood agar (CBA) plates containing 5% horse blood and Dent’s antibiotic supplement. When appropriate, antibiotic selection in *H. pylori* was carried out by supplementing media with chloramphenicol or streptomycin at a final concentration of 10 μg/ml. *Escherichia coli* DH5α was grown in Luria-Bertani broth. When necessary, antibiotics were added to the following final concentrations: ampicillin, 100 μg/ml; and chloramphenicol, 20 μg/ml.
Construction of pDifWT-RC

The construction of pDifWT-RC has been described elsewhere [12]. Plasmid pDifWT-RC is a plasmid that contains two direct repeats of the H. pylori dif sequence (\textit{difH}: TCATTTAGTTATGAAAACGTGCACTTTTCAAACCTTTAAAT) separated by the counterselectable cassette, \textit{rpsL-cat}, and flanked on either end by \textit{BamHI} restriction sites.

Construction of pUreA-DifRC for \textit{ureA} deletion

Two 1-kb DNA fragments upstream and downstream of the \textit{ureA} open reading frame (ORF) (HP0073) were amplified using primers ureA1 and ureA2, and ureA3 and ureA4. These two DNA fragments were then joined together by strand overlapping extension PCR (SOE PCR) [13] and amplified using primers ureA1 and ureA4 to give a 2-kb DNA fragment containing \textit{ureA} flanking regions separated by a unique \textit{BamHI} site. This fragment was treated with \textit{Taq} DNA polymerase and ligated into pGem®-T Easy Vector (Promega) to give pUreA-prep. The \textit{difH} flanked \textit{rpsL-cat} cassette was liberated from pDifWT-RC by \textit{BamHI} digest and cloned into the \textit{BamHI} site of plasmid pUreA-prep to give plasmid pUreA-difH-RC-F and pUreA-difH-RC-R, which differ only in the orientation of the \textit{difH-rpsL-cat-difH} insert.

Construction of pComB4-difH-RC for \textit{comB4} deletion

DNA fragments encompassing the flanking regions of \textit{comB4} were amplified from X47 genomic DNA using primer pairs comB41 and comB42, and comB43 and comB44. These fragments were joined together by SOE PCR and amplified with primers comB41 and comB44. The resulting 1.4-kb PCR product was cloned into pCR®2.1 (Invitrogen), liberated by \textit{EcoRI} digestion and subsequently cloned into pGem®-T Easy vector also digested with \textit{EcoRI} to generate
pComB4-prep. The *difH* flanked *rpsL-cat* cassette was liberated from pDifWT-RC by *BamHI* digest and cloned into the *BamHI* site internal to the *comB4* flanking regions of pComB4-prep to generate pComB4-difH-RC.

**Construction of p1031-difH-RC for *HPG27_1031* deletion**

Two DNA fragments, 900-bp upstream and 700-bp downstream of the *HPG27_1031* ORF were amplified from G27 genomic DNA using primers 1031-1 and 1031-2, and 1031-3 and 1031-4. The two DNA fragments were joined by SOE PCR to give a 1.6-kb fragment containing the DNA regions flanking *HPG27_1031* separated by a unique *BamHI* site. This PCR product was treated with *Taq* DNA polymerase and ligated into pGem®-T Easy Vector to give p1031-prep. The *difH* flanked *rpsL-cat* cassette was liberated from pDifWT-RC by *BamHI* digest and cloned into the unique *BamHI* site of p1031-prep to give p1031-difH-RC.

**Construction of pVacA-difH-RC for *vacA* deletion**

Two 1-kb DNA fragments upstream and downstream of the *vacA* ORF (HP0887) were amplified from G27 genomic DNA using primers vacA1 and vacA2, and vacA3 and vacA4. The two DNA fragments were joined by SOE PCR to give a 1.9-kb fragment containing the DNA regions flanking *vacA* separated by a unique *BamHI* site. This PCR product was treated with *Taq* DNA polymerase and ligated into pGem®-T Easy Vector to give pVacA-prep. The *difH* flanked *rpsL-cat* cassette was liberated from pDifWT-RC by *BamHI* digest and cloned into the unique *BamHI* site of pVacA-prep to give pVacA-difH-RC.
One-step clean deletion by Xer-cision in *H. pylori*

*H. pylori* strain 26695, made resistant to streptomycin by mutation in its normal *rpsL* (26695^Str^), was transformed to Cm' using pUreA-difH-RC-F and pUreA-difH-RC-R to generate strains 26695^Str^ ureA::difH-rpsL-cat-F and 26695^Str^ ureA::difH-rpsL-cat-R, respectively. The selection cassette was maintained in the transformants by continuous passage on chloramphenicol plates. Loss of the selection cassette by “Xer-cision” was selected for by sub-culturing transformants onto streptomycin plates. Single Str' colonies of 26695^Str^ ΔureA::difH-F and 26695^Str^ ΔureA::difH-R appeared after three days. Chromosomal DNA of all the clones was checked for the correct allelic replacement and for dif mediated excision of *rpsL-cat* using primers ureA1 and ureA4. The *comB4* mutation in strain X47 and the *HPG27_1031* and *vacA* mutations in strain G27^Str^ were made similarly. Strain X47, naturally streptomycin resistant [14], was transformed to Cm' using pComB4-difH-RC. Colonies growing on chloramphenicol selection plates were directly sub-cultured onto plates supplemented with streptomycin. Single colonies of Str' X47 Δ*comB4::difH*, arising after three days of incubation, were sub-cultured onto non-selective plates and chromosomal DNA was checked for the loss of *comB4*, using primers comB41 and comB44. Mutations in strain G27 were made using a strain made resistant to streptomycin by mutation in its normal *rpsL* (G27^Str^). Strain G27^Str^ was transformed to Cm' using p1031-difH-RC or pVacA-difH-RC. Colonies growing on chloramphenicol selection plates were directly sub-cultured onto plates supplemented with streptomycin. Single colonies of Str' G27^Str^ Δ*HPG27_1031::difH* were sub-cultured onto non-selective plates and chromosomal DNA was checked for the loss of *HPG27_1031* using primers 1031-1 and 1031-4, while single colonies of Str' G27^Str^ Δ*vacA::difH* were sub-cultured onto non-selective plates and chromosomal DNA was checked for the loss of *vacA* using primers vacA1 and vacA4.
**Growth curves**

Fresh cultures, passaged the day before, were resuspended in Heart Infusion broth (HI). 5 ml of HI containing 10% NCS and Dent (Oxoid) was inoculated with 100 μl of a stock inoculum standardized to OD\textsubscript{600} = 2. Growth studies were performed without any prior adaptation of \textit{H. pylori} strains to liquid media. Growth was measured every 3 to 12 h for up to 36 h. Each experiment was done in triplicate and repeated at least twice.

**Results**

The \textit{difH}/XerH site-specific recombination system for chromosome dimer resolution during DNA replication and cell division was recently identified and functionally characterized in \textit{H. pylori} [12,15]. This enabled the development of Xer-cise in \textit{H. pylori} for the construction of deletion mutants and the subsequent removal of the selectable marker in a single transformation step. Markerless gene deletions were obtained by natural transformation and homologous recombination of the Xer-cise cassette containing an \textit{rpsL-cat} counterselection marker flanked by \textit{difH} sites and neighboring homologous sequences of the target gene. Figure 1 summarises the experimental steps to perform Xer-cise in \textit{H. pylori} i) SOE PCR amplification of the flanking sequences of the target locus (with a unique \textit{Bam}HI restriction site between the flanks) and \textit{Eco}RI cloning of the resulting DNA fragment ii) cloning the counterselection \textit{rpsL-cat} marker flanked by \textit{difH} sites between the genomic flanks in the unique \textit{Bam}HI restriction site iii) natural transformation of \textit{H. pylori} with the Xer-cise cassette, homologous recombination at the target locus and selection of Cm\textsuperscript{r} transformants iv) selection on streptomycin-containing medium to isolate Str\textsuperscript{r} clones harboring a markerless deletion as a consequence of XerH-mediated excision of the \textit{rpsL-cat} cassette flanked by \textit{difH}. 
One-step transformation deletion of *ureA*

Two Xer-cise cassettes were constructed, a *ureB* colinear (Xer-cise-F) and anti-colinear (Xer-cise-R) to the *rpsL-cat* marker flanked by *difH* and homologous sequences upstream and downstream of *ureA*. To replace *ureA* with the Xer-cise cassettes by homologous recombination, natural transformation was performed using *H. pylori* 26695Sta strain harbouring a mutated *rpsL* allele conferring streptomycin resistance. Chloramphenicol resistant (streptomycin sensitive) transformants were selected (Figure 1). XerH-mediated excision of the *difH* flanked counterselection marker led to the loss of the dominant wild-type *rpsL* allele present in the counterselection cassette and restoration of streptomycin resistance (Figure 1). Thus, chloramphenicol resistant transformants were plated onto streptomycin selective plates to isolate clones that underwent the *difH*/XerH-mediated excision of the counterselection cassette, resulting in a markerless deletion of *ureA*.

After the chloramphenicol selection, diagnostic PCR was performed on genomic DNA isolated from four and five clones obtained with the Xer-cise-F and Xer-cise-R cassette, respectively. The corresponding integration of the counterselection cassettes at the *ureA* locus was confirmed for the two orientations in all the clones based on the detection of the 3-kb full length PCR product (Figure 2A, *dif-RC-F* and *dif-RC-R*, respectively). Upon streptomycin counterselection, a 1.5-kb reduction in size of the PCR fragment confirmed *difH*-mediated excision of the counterselection cassette, irrespective of the *difH*-rpsL-cat-*difH* orientation, (Figure 2A, *dif-F* and *dif-R*, respectively and data not shown). Finally, genomic DNA sequencing at the *ureA* locus of streptomycin resistant clones confirmed the markerless deletion of *ureA*, loss of the
counterselection marker and the presence of a scar sequence exactly matching that of difH (data not shown).

Xer-cise at other loci and in other genetic backgrounds

To generalize the use of the Xer-cise technique in H. pylori, two other genetic backgrounds were tested for the construction of markerless deletions, i.e., H. pylori G27Str and X47 strains were used as recipient strains to delete HPG27_1031, vacA and comB4, respectively. The corresponding Xer-cise cassettes were constructed as described in the material and methods; selection of transformants on chloramphenicol followed directly by selection on streptomycin was performed to isolate recombinant strains carrying markerless mutations. As shown in Figure 2B, diagnostic PCR for deletion of HPG27_1031 in G27Str revealed that after sequential antibiotic selection all the streptomycin resistant clones had lost the counterselection marker. The same observation was found for comB4 deletion in X47 (Figure 2C) and for vacA deletion in G27Str (Figure 2D). These results demonstrate the utility of the Xer-cise technique in H. pylori for the construction of markerless deletions in different H. pylori strains.

Low frequency of excision at ectopic difH sites

Without streptomycin counterselection, the recombination frequency at difH sites directly influences the number of clones that need to be screened after passage on antibiotic-free medium to obtain chloramphenicol susceptible clones corresponding to unmarked deletion mutants. The feasibility of using Xer-cise without counterselection (i.e., in strains without mutated rpsL allele conferring streptomycin resistance) was assessed by evaluating the frequency of XerH recombination at difH sites at the ureA locus. Chloramphenicol resistant cells, harbouring the
inserted Xer-cise-F cassette at the ureA locus, were grown for 3 days in non-selective medium (no chloramphenicol), streaked out for single colony isolates, and 100 colonies were then tested for streptomycin/chloramphenicol resistance/susceptibility phenotypes. Figure 3 shows a trend for an increase in difH recombination frequency over time that ranged from 1% to 5% after three days of incubation. Although, Xer-cision frequency is low, it would enable the recovery of unmarked deletion by replica plating and without counterselection.

Multiple difH sites per chromosome do not interfere with bacterial fitness

Repeated rounds of Xer-cise for the construction of multiple deletion mutants in the same strain leads to the presence of more than one difH site per chromosome and could theoretically promote genetic instability by chromosomal deletions. To test whether multiple chromosomal difH sites in a strain could promote chromosomal deletions or interfere with bacterial fitness, a triple gene deletion mutant was constructed in the strain 26695Str. The ureA, vacA and HP0366 (corresponding to HPG27_1031 in G27) unmarked triple deletion mutant was constructed by natural transformation using the corresponding Xer-cise cassettes. Sequential deletion of ureA, vacA and HP0366 was achieved by removal of the selectable marker at each step, allowing repeated rounds of selection on chloramphenicol-containing medium and counterselection on streptomycin-containing medium. Diagnostic PCRs confirmed the deletion of the three genes as well as the loss of all three counterselection cassettes after the sequential antibiotic selection (data not shown). Of note, the triple knockout displayed a slightly higher growth rate in liquid medium compared to wild-type (Figure 4), suggesting that 3 additional difH sites per chromosome, at least for the combination of the three selected loci, did not interfere with viability or bacterial fitness.
Discussion

Xer-cise is a one-step transformation deletion technique [11] based on the dif/Xer system for the construction of markerless mutations. This technique was adapted to *H. pylori* by taking advantage of the recent functional demonstration of the single recombinase difH/XerH system for the resolution of chromosome dimers in *H. pylori* [12]. The removal of the counterselection cassette after its integration into the genome by homologous recombination was achieved by flanking it with *difH* sequences that promote XerH-mediated excision from the chromosome. The resulting chromosomal recombination product is a markerless deletion harboring a scar corresponding to the *difH* sequence.

Once integrated in the genome, the Xer-cise cassette can be maintained by keeping the positive chloramphenicol selection or counterselected when using the negative streptomycin selection. Thus, the combination of Xer-cise with counterselection allows for the construction of a markerless mutation in one transformation step instead of two, in the case of the replacement of the counterselection cassette by natural transformation and homologous recombination [9]. The efficiency of XerH recombination of ectopic tandemly repeated *difH* sites at the *ureA* locus without counterselection ranged between 1-5% after 48 h. This result is in line with the recombination frequency of 1-6.3% in *E. coli* [11] and of about 4% in *H. pylori* [12] after 48 h, but much less than the reported 99% in *E. coli* when the tandemly repeated *dif* sites are located near the chromosome terminus, the original *dif* location required for efficient recombination [16]. This result confirms that chromosomal position of paired *dif* sites far away from the natural *dif* near the chromosome terminus leads to inefficient Xer recombination in *H. pylori* [12] and in *E. coli* [17]. Much higher frequency were observed with the RipX/CodV system in *B. subtilis*.
after 48 h ranging from 41.3-68.7 % [11] suggesting that this system is more efficient than Xer-cise, independently of the chromosomal position. Nevertheless, the reported low XerH recombination frequency allows the recovery of unmarked deletion by replica plating and testing for the loss of chloramphenicol resistance. Of note, Xer-cise combined with \( rpsL \) counterselection led to a 100% excision in the streptomycin resistant clones, although only a fraction of the resulting clones were tested since several clones are usually sufficient for most genetic studies. The high efficiency of Xer-cise (100% for the four deletion tested in this study) compared to the varying efficiency (17% to 90%) of the \( rpsL-cat \) counterselection cassette replacement by homologous recombination [9], suggests that Xer-cise efficiency is mostly dependent of Xer-recombination and is less affected by gene conversion of the \( rpsL \) alleles and other effects such as transformation efficiency or local effects on homologous recombination.

Our results with three markerless deletions in three unrelated \( H. pylori \) strains suggest that an additional ectopic \( difH \) site does not result in adverse effects on chromosome segregation. Although the latter observation is in line with a previous report of Xer-cise in \( Bacillus subtilis \) and \( Escherichia coli \) [11], multiple \( dif \) sites insertions or an insertion too close from the natural \( dif \) site at the chromosome terminus might promote chromosomal deletions of the intervening DNA between two \( difH \) sites. A triple (\( ureA \), \( vacA \) and \( HP0366 \)) deletion mutant was successfully constructed using Xer-cise and was viable. Unexpectedly, a slightly higher growth rate rather than a growth defect was observed for the triple mutant strain. The latter phenotype may reflect a reduced metabolic load due to the lack of expression of the abundant urease enzyme. Figure 5 shows the relative positions of \( ureA \), \( vacA \), \( HP0366 \) and \( difH \). The smallest possible chromosomal deletion between the natural \( difH \) and the additional \( difH \) at the \( vacA \) locus is of about 220,000 bp. This region contains several potential essential genes [2] and its deletion
would thus be lethal. The viability of the triple mutant fits the observed low recombination frequency between two difH sites far away from each other and not positioned in their natural sequence context (the chromosome terminus) and suggests a general low recombination rate between ectopic difH sites in H. pylori. The almost normal growth rate of the triple mutant indicates that additional difH sites in the genome do not affect H. pylori fitness and demonstrates the applicability of Xer-cise for constructing multiple unmarked gene deletions in H. pylori.

To date more than a dozen markerless gene deletion mutants have been constructed with Xer-cise in our laboratory (Li, Gauntlett, Liao and Benghezal unpublished data) validating the simplicity and efficiency of this technique. Furthermore, new possibilities such as its use with a transposon for a one-step protein in frame tagging have been successful in H. pylori (Sehnal and Benghezal unpublished data), due to the presence of two uninterrupted open reading frames, out of the possible six, in the difH sequence (Figure 6).

To conclude, Xer-cise is a novel genetic tool to study H. pylori pathogenesis. This technique reduces the number of transformations and decreases the workload of subsequent clone screening; a single transformation step followed by simple and direct selection for loss of the cassette halves the time required to generate clean deletion mutants.

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References


Figures and figure legends

Figure 1

i  

\[\text{Flank A} \quad \text{Flank B}\]

\[\text{EcoRI} \quad \text{BamHI} \quad \text{EcoRI}\]

\[\text{pXX-prep}\]

ii  

\[\text{difH} \quad \text{rpsL-cat} \quad \text{difH}\]

\[\text{difH-rpsL-cat-difH}\]

\[\text{from pDiffWT-RC}\]

\[\text{EcoRI} \quad \text{BamHI} \quad \text{BamHI} \quad \text{EcoRI}\]

\[\text{Flank A} \quad \text{difH} \quad \text{rpsL-cat} \quad \text{difH} \quad \text{Flank B}\]

\[\text{pXX-difH-RC}\]

iii  

\[\text{Flank A} \quad \text{difH} \quad \text{rpsL-cat} \quad \text{difH} \quad \text{Flank B}\]

\[\text{Natural transformation}\]

\[\text{geneXX}\]

\[\text{Homologous recombination}\]

\[\text{Cm}^S, \text{Str}^f\]

\[\text{Selection on chloramphenicol}\]

\[\text{Flank A} \quad \text{difH} \quad \text{rpsL-cat} \quad \text{difH} \quad \text{Flank B}\]

\[\text{Cm}^r, \text{Str}^s\]

iv  

\[\text{Selection on streptomycin}\]

\[\text{Xer-cision}\]

\[\text{Flank A} \quad \text{difH} \quad \text{Flank B}\]

\[\text{Cm}^S, \text{Str}^f\]
The construction of a markerless deletion in *H. pylori* mediated by the difH/XerH recombination system involves four steps including a single natural transformation of the Xer-cise cassette. i) SOE PCR amplification of the flanking sequences of the target locus (with a unique BamHI restriction site between the flanks) and EcoRI cloning of the resulting DNA fragment ii) cloning the counterselection *rpsL-cat* cassette flanked by difH sites between the flanks at the unique BamHI restriction site iii) natural transformation of *H. pylori* with the Xer-cise cassette, homologous recombination at the target locus and selection for chloramphenicol resistant transformants iv) selection on streptomycin medium to isolate clones harboring a markerless deletion as a consequence of XerH mediated excision of the *rpsL-cat* cassette flanked by difH.
Figure 2. Xer-cise mediated construction of 4 markerless deletions in three different H. pylori strains.

(A) ureA deletion in strain 26695ΔureA::
dif-RC-F dif-RC-R dif-F dif-R WT

(B) WT G27 ΔHPG27_1031::difH

(C) WT X47 ΔcomB4::difH

(D) WT G27 ΔvacA::difH

Figure 2. (A) ureA deletion in strain 26695ΔureA:: Using cassettes with opposite dif-rpsL-cat-dif orientation, Xer-cise-F and Xer-cise-R. Insertion of either Xer-cise-F or Xer-cise-R gave rise to chloramphenicol resistant clones. Diagnostic PCR of four clones obtained with Xer-cise-F (dif-RC-F) and five clones obtained with Xer-cise-R (dif-RC-R) produced the expected 3-kb PCR product, demonstrating the replacement of ureA with rpsL-cat, irrespective of the orientation of difH-rpsL-cat-difH. Counterselection of chloramphenicol resistant clones on streptomycin plates led to a reduction of the diagnostic PCR product by 1.5-kb in the two
clones tested for the Xer-cise-F (dif-F) and Xer-cise-R (dif-R) cassettes, demonstrating XerH-mediated excision of *rpsL-cat*. Wild-type strain gave a PCR product of about 2-kb. (B) *HPG27_1031* deletion in *H. pylori* strain G27Str. XerH-mediated excision corresponding to the desired markerless deletion was detected by a shift in size of the PCR product from 3-kb to 1.5-kb in all six streptomycin resistant clones tested. (C) *comB4* deletion in *H. pylori* strain X47. XerH-mediated excision corresponding to the markerless deletion of *comB4* was detected by a shift in size of the PCR product from 3-kb to 1.5-kb in all streptomycin resistant clones tested. (D) *vacA* deletion in *H. pylori* strain G27Str. XerH-mediated excision corresponding to the markerless deletion of *vacA* was detected by a shift in size of the PCR product from 5.8-kb to 2-kb in all five streptomycin resistant clones tested.
Figure 3. XerH recombination frequencies for Xer-cise cassette located at ureA locus.

Cells were grown on non-selective medium for 3 days, re-streaked for single colonies, and 100 colonies per time point were tested for retention or loss of rpsL and cat genes by replica plating to streptomycin and to chloramphenicol containing media. Experiments were performed in triplicates; horizontal bars indicate means and standard deviation of the percentage of recombinants.
Figure 4

Growth curves of wild-type and triple mutant constructed by Xer-cision.

Growth curves of *H. pylori* in liquid medium. Cells were grown in HI liquid medium supplemented with 10% NCS under microaerobic conditions with agitation. The optical densities (OD$_{600}$) of wild-type (WT) and triple (*ureA, vacA and HP0366*) deletion mutant were measured in triplicate for up to 40 h, standard deviation is indicated. The triple mutant growth rate was slightly higher than the WT, suggesting that multiple *difH* sites do not negatively affect bacterial fitness.
Figure 5. Positions of $difH$ and gene deletions in the $H. pylori$ 26695 genome sequence.

The circular $H. pylori$ genome is presented with the positions (bp) of the $difH$, $ureA$, HP0366 and $vacA$ loci.
Figure 6. *difH* open reading frame.

The 40-bp sequence of *difH* were analysed for the presence of open reading frames in the six possible frames. Two uninterrupted open reading frames (+1 and +2) enable Xer-cise in frame protein insertion tagging.
Table 1. Plasmids and bacterial strains used in this study.

<table>
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<tr>
<th>Plasmid or strain name</th>
<th>Description</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td>pDifWT-RC</td>
<td><em>rpsL-cat</em> cassette flanked by <em>difH</em></td>
<td>[12]</td>
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<tr>
<td>pUreA-prep</td>
<td>pGem-T Easy vector containing two 1-kb sequences flanking HP0073, separated by a <em>BamHI</em> restriction site</td>
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<td>pUreA-difH-RC-F</td>
<td>Derivative of pUreA-prep, with <em>difH-rpsL-cat-difH</em> inserted between <em>ureA</em> flanking sequences at <em>BamHI</em> site, orientated in the forward direction</td>
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<td>pUreA-difH-RC-R</td>
<td>Derivative of pUreA-prep, with <em>difH-rpsL-cat-difH</em> inserted between <em>ureA</em> flanking sequences at <em>BamHI</em> site, orientated in the reverse direction</td>
<td>This work</td>
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<td>pComB4-prep</td>
<td>pGem-T Easy vector containing sequences flanking <em>comB4</em> (750-bp, 650-bp), separated by <em>BamHI</em> restriction site</td>
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<td>p1031-prep</td>
<td>pGem-T Easy vector containing sequences flanking <em>HPG27_1031</em> (900-bp, 700-bp), separated by <em>BamHI</em> restriction site</td>
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<td>pVacA-difH-RC</td>
<td>Derivative of pVacA-prep, with <em>difH-rpsL-cat-difH</em> inserted between <em>vacA</em> flanking sequences at <em>BamHI</em> site</td>
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*H. pylori* strain:

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<th>Strain</th>
<th>Description</th>
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<tr>
<td>26695^Str^</td>
<td>Streptomycin resistant</td>
<td>[9]</td>
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<td>26695^Str^ <em>ureA::difH-rpsL-cat-F</em></td>
<td><em>ureA</em> replaced with <em>difH-rpsL-cat-difH</em> selectable marker, forward orientation</td>
<td>This work</td>
</tr>
<tr>
<td>26695^Str^ <em>ureA::difH-rpsL-cat-R</em></td>
<td><em>ureA</em> replaced with <em>difH-rpsL-cat-difH</em> selectable marker, reverse orientation</td>
<td>This work</td>
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### Plasmid or strain name

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<td><strong>26695</strong>&lt;sup&gt;Str&lt;/sup&gt; Δ&lt;sup&gt;ureA&lt;/sup&gt;::&lt;sup&gt;difH&lt;/sup&gt;-R</td>
<td>ureA replaced with single difH, reverse orientation</td>
<td>This work</td>
</tr>
<tr>
<td><strong>26695</strong>&lt;sup&gt;Str&lt;/sup&gt; triple knockout</td>
<td>ureA replaced with single difH, HPG27_1031 replaced with single difH and HP0887 replaced with single difH</td>
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<tr>
<td>X47</td>
<td>Naturally streptomycin resistant</td>
<td>[14]</td>
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<tr>
<td>X47 Δ&lt;sup&gt;comB4&lt;/sup&gt;::&lt;sup&gt;difH&lt;/sup&gt;</td>
<td>HP0017 replaced with single difH</td>
<td>This work</td>
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<td><strong>G27</strong>&lt;sup&gt;Str&lt;/sup&gt; Δ&lt;sup&gt;HPG27_1031&lt;/sup&gt;::&lt;sup&gt;difH&lt;/sup&gt;</td>
<td>HPG27_1031 replaced with single difH</td>
<td>This work</td>
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<tr>
<td><strong>G27</strong>&lt;sup&gt;Str&lt;/sup&gt; Δ&lt;sup&gt;vacA&lt;/sup&gt;::&lt;sup&gt;difH&lt;/sup&gt;</td>
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### Table 2. Primers used in this study.

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<tr>
<td>ureA1</td>
<td>CCAAGGCTAGTGAATTGAATGTC</td>
<td>Construction of pUreA-prep</td>
</tr>
<tr>
<td>ureA2</td>
<td>CATAGGATCCGAGTTTCTATTCTCTATTCTTAAGTG</td>
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</tr>
<tr>
<td>ureA3</td>
<td>GAGAATAAGATGAAACTCGGATCTATGTAATTAAAGAGAATAGC</td>
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</tr>
<tr>
<td>ureA4</td>
<td>CGCGATGATTGATTGCAGAAGGAG</td>
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<tr>
<td>comB41</td>
<td>GAAGGGTTTTGATGTCCGCTC</td>
<td>Construction of pComB4-prep</td>
</tr>
<tr>
<td>comB42</td>
<td>CATGGGCATCCGCTCTGCTTAATGGGTGTAAG</td>
<td></td>
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8 General discussion

_H. pylori_, is an ancient member of the human microbiota that has coevolved with humans to dominate the gastric niche. Infection causes chronic active gastritis which may develop into peptic ulceration or, more rarely, gastric adenocarcinoma. However, the majority of infected individuals (80-90%) carry and transmit _H. pylori_ without any symptoms of disease. Understanding when colonization by this bacterium is beneficial and why some infections result in disease is critical to a better understanding of the role of microbiota in human health and disease as a whole and has important applications in developing strategies to improve quality of life.

Deletion mutants in combination with the use of animal models have been instrumental in the study of _Helicobacter pylori_ pathogenesis. However, the use of gene deletion limits the study to loss-of-function knockout mutants. This approach runs the risk of selecting for mutants that have adapted to the deletion genotype by secondary mutations and does not allow for investigating whether a specific gene is necessary to maintain the infection after the initial colonization step or whether it is necessary for the entire infection cycle. Therefore conditional knockouts, based on an inducible expression of the target gene, are better suited to study the temporal requirement of specific genes during infection and in physiological settings. This approach is of particular importance for the study of _H. pylori_ pathogenesis as infection is persistent and clinical diseases develop after many years of chronic inflammation and epithelial damage. The lack of suitable genetic systems to control gene expression in _H. pylori_ has been a handicap in this area of research.

The _tet_ repressor (TetR) inducible system has been extensively studied and used with great success to modulate gene expression in animal infection models for three bacterial pathogens. This study adapted the _tet_-system for use in _H. pylori_ and generated new genetic tools that can be used to elucidate the different roles that specific genes play in _H. pylori_ pathogenesis and in disease progression. It also confirmed that _tet_-regulation is a powerful genetic tool for studying _H. pylori_ infection in the context of an intact gastric environment. This study utilized the newly adapted _tet_-system to modulate the expression of two virulence factors, cholesterol α-glucosyltransferase (CGT) and urease, _in vivo_ to study their role in persistent infection.
8.1 Adaptation of tet-regulation to \textit{H. pylori}

To date, tet-regulated expression systems have been developed from several different Tc resistant determinants and used in 20 different bacterial species. The main obstacle to translating tet-regulation to other bacterial species has been the functional optimization of the tet-responsive promoter for the host bacterium. In this study the well characterized \textit{ureA} promoter of \textit{H. pylori} was used to develop two sets of \textit{H. pylori} tet-promoters, \textit{uPtetO(1-3)} and \textit{urePtetO(I-V)}, which were demonstrated to regulate target gene expression in \textit{H. pylori} conditional mutants in a tetracycline dependent manner.

The \textit{uPtetO1} tet-promoter could be regulated over an 80-fold range by anhydrotetracycline (ATc) in \textit{H. pylori} and, although it is not nearly as large as the induction range that can be achieved in some \textit{E. coli} tet-systems (2500- to 5000-fold) (Lutz and Bujard, 1997), the regulatory range achieved in this study is comparable to the first generation tet-regulations systems adapted to other bacteria such as \textit{B. subtilis} (100-fold), \textit{S. aureus} (50- to 100-fold), \textit{M. smegmatis} (170-fold) and \textit{S. pneumonia} (5-fold) (Ehrt et al., 2005; Geissendorfer and Hillen, 1990; Ji et al., 1999; Stieger et al., 1999). The regulatory range of the \textit{urePtetO} tet-promoters was equally large, enabling the generation of conditional urease mutants to study the functional role of \textit{H. pylori} urease during persistence. This study also demonstrated that adjustments to regulatory windows could be made to suit target gene expression and was accomplished by adjusting \textit{tetR} or \textit{revTetR} expression by way of different promoters.

Interestingly, response to tet-regulation was relatively slow in \textit{H. pylori} compared to other bacterial species. Complete induction of UreB and GFP expression in \textit{H. pylori} required 12 h and 16 h respectively, which is about 4 times slower than tet-induction in \textit{E. coli} and \textit{B. subtilis} (3-4 h) (Geissendorfer and Hillen, 1990; Lutz and Bujard, 1997). This slow induction profile is likely an inherent characteristic of \textit{H. pylori} as a similarly delayed induction profile was described for the \textit{lacI} conditional expression system in \textit{H. pylori} (Boneca et al., 2008). The leisurely growth rate of \textit{H. pylori} (2-4 h) (Baltrus and Guillemin, 2006; Vega et al., 2003) may explain the delayed response as tet-induction in \textit{M. tuberculosis}, a very slowly replicating species, takes days (Ehrt et al., 2005). However, this explanation is incomplete as the generation time of \textit{M. smegmatis} (2-3 h) is similar to that of \textit{H. pylori} yet complete tet-induction in this species can be achieved significantly faster (4 h) (Ehrt et al., 2005; Snapper et al., 1990).
8.2 Tet-regulation as a tool to study *H. pylori* pathogenesis and persistence

This study demonstrated that the *tet*-system developed for *H. pylori* is stable in replicating bacteria *in vivo* and that it can be used to regulate *H. pylori* gene expression in the mouse model. To that end the *tet*-system was applied in this study to investigate the role of two *H. pylori* virulence factors, CGT and urease, in the mouse infection model.

It has been well established, with the use of deletion mutants, that urease is an important colonization factor, however as these mutants are unable to establish infection it could only be inferred from *in vitro* studies that urease may be important for *H. pylori* persistence. The mouse infection experiments using *tet*-conditional X47 urease mutants conducted in this study provides the first direct evidence that urease is required to establish and maintain infection even after *H. pylori* has reached the relatively neutral environment of the gastric mucosa. This study also demonstrated that there is a strong selective pressure on *H. pylori* to continuously express urease in order to survive in its gastric niche.

Conditional gene expression systems also permit the titration, both up and down, of target gene expression under culture conditions or in animal models, and thereby provide a powerful approach to gaining quantitative data on the functional importance of a gene product to either bacterial growth or virulence. This utility was made particularly evident in *tet*-regulation of CGT expression. Despite failing to generate a conditional *cgt* mutant, this study demonstrated that CGT is not abundantly expressed and that perturbations in CGT expression affect the ability of *H. pylori* to establish infection. The results of this study suggest that the controlled expression of *cgt* by *H. pylori* is important for the bacterium’s fitness *in vivo* and highlight the importance of proper promoter replacement in complementation studies. The *cgt* experiments described in this study confirmed that the uPtetO based *tet*-system can be used to complement and regulate *H. pylori* genes however they also demonstrated that the system is currently limited to gene products that are expressed more abundantly in the bacterium.
8.3 Future directions to broaden the utility of tet-regulation in *H. pylori*

Further refinement to the *H. pylori* tet-system is necessary to broaden the utility of tet-regulation in *H. pylori* and to meet the needs of individual researchers. No single conditional expression system developed to date can accommodate the wide range of gene expression levels that occur in bacteria. Though established *E. coli* tet-systems can achieve the greatest regulatory range of any conditional expression system, tet-systems that permit higher levels of protein do not provide the most stringent repression, while tet-systems that permit very tight gene repression consequently have significantly lower induction maxima (Lutz and Bujard, 1997). The same principle has been observed in tet-systems developed for other bacterial species (Geissendorfer and Hillen, 1990; Kamionka et al., 2005). Therefore future studies should focus on developing a small library of well-regulated *H. pylori* tet-promoters that differ in their induced activities, so that an appropriate promoter can be selected for the target gene of interest. Several modifications can be made to the *H. pylori* tet-system described in this study to generate second generation tet-promoters with regulation properties that are suitable for regulating gene products that are found in very low abundance in *H. pylori* (discussed in Chapter 5).

The chromosome integration strategy used in this study provides genetic stability and the absence of resistance markers, and should be applicable to a number of different *H. pylori* strains. However, the time required for strain construction can be lengthy. One methodology for reducing construction time is to use counterselection cassettes in conjunction with gene synthesis services, which have become increasingly more economical over the last few years. Another alternative is to generate a construct, consisting of *ptetR* and *uPtetO-geneX* arranged in such a way as to have divergent polarities, which can be cloned into a shuttle vector as a single functional unit. This may be an attractive option for studies using strains which readily take up shuttle plasmids as a plasmid-based tet-regulation system will greatly decrease strain construction time and allow for greater levels of protein expression than can be achieved using chromosomally based systems.
For studies in which rapid gene silencing is the goal, the TetR system may be less ideal for tet-regulation as the inducer has to be removed to achieve gene silencing and this can be delayed due to accumulation of the inducer in the bacterial cell or in animal tissue. In such cases, studies would benefit from the revTetR system, where gene expression can be turned off by the addition of the tetracycline co-repressor. However in this study, revTetR was less efficient than TetR at silencing gene expression in *H. pylori* and this was shown to be due to the lower steady state levels of revTetR compared to TetR. This problem has been reported for several other bacterial species in which the revTetR system has been employed and strategies which overcome this limitation have consequently led to the development of efficient revTetR systems for these organisms (Ehrt et al., 2005; Guo et al., 2007; Kamionka et al., 2005; Stary et al., 2010). Future directions for improving gene regulation in *H. pylori* by the revTetR system should focus on strategies that increase the steady state levels of revTetR (discussed in Chapter 4).

An alternative strategy to using revTetR for rapid gene silencing is to use TetR to regulate the expression of antisense RNA, so that the addition of inducer effectively results in gene silencing. Recent characterization of the *H. pylori* primary transcriptome has revealed that *H. pylori* has genome-wide antisense transcription which may be used by the bacterium to regulate gene expression (Sharma et al., 2010). Therefore the combination of antisense RNA expression with tet-regulation can be exploited in *H. pylori* for minimally invasive target gene control. Such a system would be well suited for the regulation of essential genes or those within operons, which are left untouched in their original locus. This regulation strategy has been used with great success to study essential genes in the *S. aureus* animal infection model (Ji et al., 1999; Ji et al., 2001) and its application for bacteria has been reviewed (Yin and Ji, 2002).

With regards to the use of tet-regulation in future *in vivo* studies, doxycycline (Dox) is a much cheaper alternative to ATc and is routinely used to regulate tet-systems in animal models. However, this study found *H. pylori* strain X47 to be relatively sensitive to Dox supplementation *in vivo* which consequently limits the maximum amount of Dox supplement that can be used to regulate *H. pylori* gene expression during infection. One way to improve the utility of the tet-system *in vivo* would be to increase *H. pylori* resistance to tetracyclines, either by mutating the 16S RNA or by introducing a
ribosome protection protein, to permit higher concentrations of Dox to build up inside the bacterium.

8.4 Other additions to the *H. pylori* genetic tool box

In the course of developing the tet-system for use in *H. pylori*, this study demonstrated the efficacy of four additional chromosomal loci, *mdaB*, *gltDH*, *trpA* and *dapB* locus, as suitable chromosomal complementation systems for *in vivo* studies. These loci supported the expression of foreign proteins and their genetic manipulation was efficient and had no discernible impact on the colonization fitness of strain X47. These four chromosomal loci are now also used routinely in Ondek Pty. Ltd. for chromosomal complementation in *H. pylori* strains G27 and B128, and may be very useful for the genetic studies of *H. pylori* strains in which plasmid complementation has proved difficult. The slow growth rate of *H. pylori* imposes significant limitations to the rate at which outcomes are achieved in *H. pylori* genetic studies. Though not directly used in this study to develop the tet-system and construct conditional *H. pylori* mutants, it is envisioned that the adaptation of Xer-cision, in combination with counterselection, to *H. pylori* (described in Chapter 7) will serve as a useful new genetic tool to expedite the construction of markerless mutations and the generation of conditional mutants in many different *H. pylori* strains (Debowski et al., 2012).

8.5 Conclusions

Model organisms such as *E. coli*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster*, have greatly contributed to our understanding of biological processes, and genetic tools have been critical to probing the molecular networks of these living systems. The lack of such tools though has been a limitation in the study of organisms such as *H. pylori*. This study has adapted new *E. coli* derived molecular biology techniques for use in *H. pylori* genetics. The use of tetracycline dependent gene regulation and XerH mediated recombination has not been previously reported for *H. pylori* or for any other members of the ε-proteobacteria. This study has adapted the tetracycline repressor inducible system for use in *H. pylori* and demonstrated that tet-regulation can be used to control *H. pylori* gene expression to study mechanisms of *H. pylori* persistence, facilitating new avenues of inquiry into
*H. pylori* pathogenesis. The different tet-promoter and tet-repressor constructs described in this study, can be combined with an appropriate chromosome position to provide three different levels of regulation for modulating and fine tuning gene expression. This chromosomally based tet-system and Xer-cise technology should be transferable to a large variety of *H. pylori* isolates with different genetic backgrounds.
9 References


pathogenic potential is correlated to cell wall lipid composition. Scand. J. Gastroenterol. 32, 445.


secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium. J. Pathol. 188, 220.


activation of cholesterol alpha-glucosyltransferase, an enzyme responsible for biosynthesis of cholesteryl-alpha-D-glucopyranoside in *Helicobacter pylori* critical for its survival. J. Histochem. Cytochem. 59, 98.


the BabA adhesin during experimental infection with Helicobacter pylori. Infect. Immun. 78, 1593.
complete genome sequence of the gastric pathogen Helicobacter pylori. Nature 388, 539.


