The Genetic Characterisation of a Carbon Starvation Gene of *Helicobacter pylori*

A thesis presented in partial fulfilment of

the requirements for the degree of

Master of Science in Microbiology

at Massey University, Palmerston North

New Zealand

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1998

Abstract

The carbon starvation gene (*csg*) of the gastric pathogen *Helicobacter pylori* was studied to elucidate the role of the gene product during the carbon starvation response. Studies were also conducted to investigate the relationship of *csg* to the glutamine permease operon (*glnP1*, *glnP2*, *glnQ*, and *glnH*), which is located directly upstream of *csg* on the *H. pylori* chromosome.

csg and *glnP1* were sequenced to determine the length of the genes, the base composition, and the location of putative promoters and regulator binding sites. The sequence information was then used to construct a *csg* mutant using a deletion:insertion strategy, whereby the promoter regions of *csg* and *glnP1* were removed and replaced with the KanΩ antibiotic resistance marker. Phenotypic analysis was carried out on this mutant in complex, defined, and semi-defined media. The plasmid carrying the *csg* mutation was also transformed into *H. pylori* Sydney strain to allow colonisation studies in mice.

A *H. pylori* strain was constructed, which would allow the study of the expression of *csg* under various environmental conditions. The promoterless chloramphenicol acetyl transferase (CAT) cassette from pCM4 was used as the reporter gene and cloned into an engineered *Bgl*II site directly downstream of *csg* to disrupt the transcription terminator. The kanamycin resistance cassette from pILL600 was also cloned into this site in a three way ligation, to allow selection of transformants in *H. pylori*.

Sequence of the *csg* mutant showed putative σ^{70} responsive -10 and -35 sequences, and a putative cAMP receptor protein (CRP) binding sequence for *csg* in the 5' sequence of *glnP1*. The CRP site implied that *csg* may be regulated by cAMP. The location of the CRP binding site also suggested that *csg* and *glnP1* were linked, which was confirmed by PCR amplification of a region containing the *csg* and *glnP1* transcription start points and the intergenic region from ten *H. pylori* strains. The same PCR amplification failed for a *H. mustelae* strain, suggesting that this conservation of sequence is specific to *H. pylori* strains.

Phenotypic analysis of the *csg* mutant showed no difference compared to the wild type when grown in complex and semi-defined medium, both of which contained peptides. However, the *csg* mutant grew more slowly than the wild type in the defined medium, which contained glucose and amino acids as the sole carbon sources.

The role of *csg* could not be determined with certainty. The results suggested that Csg may scavenge for carbon sources to help the cell escape carbon starvation. This thesis work has shown *csg* to be non-essential *in vitro*. However, a non-functional *csg* gene *in vivo* may impair transmission, when carbon sources are lacking, but the mutation would be silent in the stomach where peptides are abundant.

Acknowledgements

I would firstly like to give a big thanks to my supervisor, Paul O'Toole, for letting me try and think for myself and then helping out when that didn't work! An especially big thanks for reading and correcting this tome – definitely no small feat!

I would also like to thank the members of the Department of Microbiology and Genetics, now part of the Institute of Molecular Biosciences, for their encouragement and support for us postgrads. Massey University also needs to be thanked, and I give thanks from the bottom of my heart for the scholarships which have helped over the past two years.

A big thank you goes to all members, past and present, of the Helipad. In particular, Tash for being a life saver on a number of occasions and also for being great as a sounding board for ideas; Steffen for technical support and the odd german lesson; Basil for being a fountain of knowledge on 80's music - we couldn't have done the Manic Medley without you; and Kirsty for being a great bench buddy. Thanks to everyone for making the last two years fun.

I must also thank my friends in the Department/Institute; Kirsty, Helen, Ross, BJ, James, Megan, Tash, and Anita for the smokos, lunches, and support.

An absolutely huge thank you to my family for their emotional and financial support over the last five years that I've been at varsity. Your encouragement has meant so much to me and I love you guys dearly.

Finally, to Ru. For your encouragement, for forcing me back to my room to study, for letting me vent about things that weren't working, for all the understanding (the non-scientific variety) and support, and for all of your love. You are my best friend and I love with all of my heart. Ta.

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1.0 Introduction

1.1 Historical Overview

Spiral bacteria, classified as spirocheates, have been observed and documented in human stomachs since 1938. Doenges (1938) observed spirocheates in 43% of 242 stomachs at necropsy, but drew no conclusions. Freedburg and Barron (1940) observed spirocheates in 37% of gastrectomy specimens but concluded that the bacteria colonised tissue surrounding benign or malignant ulcers as non-pathogenic opportunists. Palmer (1954) studied 1140 gastric biopsy specimens and, finding no spirocheates, declared that previous reportings to have been oral contaminants which multiplied in post-mortem specimens or close to ulcers. However, Palmer did not use silver staining as used in previous studies and which stains the spiral bacteria most effectively (Warren and Marshall, 1983).

Spiral shaped gastric bacteria have been well documented in other mammals, and are thought to be commensals. The spirocheates observed in human gastric biopsy samples were largely overlooked before 1980, as they were considered to be the same as those gastric bacteria of other mammals. In 1983, however, Warren and Marshall described in detail the spiral shaped bacteria from human gastric biopsy samples. The bacteria that Warren and Marshall (1983) had observed were described as curved bacilli resembling *Campylobacter jejuni* by light microscopy.

Due to the resemblance to *Campylobacter jejuni*, Campylobacter isolation techniques were used to culture the bacterium. Chocolate agar plates were incubated in microaerophilic

conditions, with growth appearing after 3-4 days as a faint transparent layer. The bacteria were shown to have smooth coats and up to five sheathed polar flagella. Warren and Marshall (1983) also noted that similar flagella were described in Vibrio bacteria, but microaerophilic vibrios were transferred to the genus *Campylobacter*. Campylobacters have a single polar flagella at one or both ends of the cells, and which is not sheathed. However, the newly isolated bacterium was finally classified as *Campylobacter pyloridis*, a name which was later amended to *Campylobacter pylori* in order to make the specific epithet grammatically correct (Marshall and Goodwin, 1987).

As investigations into *C. pylori* continued between 1982 and 1989, it became apparent that the bacterium was not of the genus *Campylobacter*. Goodwin *et al* (1989) demonstrated that *C. pylori* differed from campylobacters in that the cellular fatty acids and major ultracellular features were quite different, that the antibiotic susceptibilities were also different, and that methylated menoquinone (MK-6), present in all campylobacters, was absent. Four studies of 5S and 16S ribosomal RNA sequences confirmed that *C. pylori* should not be included in the genus *Campylobacter* (Goodwin *et al*, 1989). These studies also indicated that the bacterium might probably be more closely related to *Wolinella succinogenes*. Further testing of five taxonomic features (ultrastructure features, cellular fatty acid profiles, respiratory quinones, growth characteristics, and enzyme capabilities) revealed that *C. pylori* should not be included in the *Wolinella* genus. It was finally concluded that *C. pylori*, while similar to *Campylobacter* and *Wolinella* sp., represented bacteria from a new genus. This genus was named *Helicobacter* and *C. pylori* was renamed *H. pylori* (Goodwin *et al*, 1989).

1.2 General features of *H. pylori*

1.2.1 Morphology of *H. pylori*

H. pylori is a Gram negative, microaerophilic bacterium, made motile by one to five sheathed polar flagella. The bacterium has two morphological forms - a rod form and a coccoid form. There is a conversion from the helical rod shape to a coccoid shape after extended incubation, during nutrient deprivation, and during exposure to antibiotics (Moshkowitz *et al*, 1994; West *et al*, 1994; Bode *et al*, 1993). The rod shape has usually been associated with infection while the coccoid shape has been suggested to be responsible for transmission (Enroth *et al*, 1995). The coccoid form of *H. pylori* has been demonstrated to exhibit higher extracellular matrix binding capabilities than the helical form, suggesting that the coccoid form could be considered as an infective form in the pathogenesis of *H. pylori* infection (Cole *et al*, 1997; Khin *et al*, 1996). The coccoid form is also the form isolated from the environment. Debate exists over whether this coccoid form represents a dormant phase or is a form associated with death. Kusters *et al* (1997) argue that the degradation of DNA and the loss of RNA observed during conversion from rods to cocci implies that the conversion is a passive process that results from cell death. Sörberg *et al* (1996) demonstrated metabolic activity in the coccoid cell and suggest that there may be two types of coccoid cell - one that is dormant and exhibits some metabolic activity, and one that represents a dead cell. Metabolic activity has also been demonstrated in coccoid cells by Bode *et al* (1993) and Nilius *et al* (1993). The studies of Andersen *et al* (1997) also supported the theory that there may be two coccoid cell types. Andersen *et al* (1997) studied the growth and morphological transformation of *H. pylori* were studied and the evidence suggested that some cocci can revert back to a helical form with full recovery of urease activity.

The ability to form coccoid cells during nutrient deprivation is not limited to *H. pylori*. Coccoid cells are also observed in cultures of *Vibrio* sp., *Campylobacter* sp, and *E. coli* (Morita, 1993; Ostling *et al*, 1993; Boucher *et al*, 1994; Lange *et al*, 1991). The coccoid cells of *Campylobacter* sp. and *Vibrio* sp. are termed viable but non-culturable, as normal laboratory culturing methods are not able to produce viable cells (Benaïssa *et al*, 1996; Oliver, 1993). *H. pylori* coccoid cells may similarly be viable but non-culturable and, if so, would signify that the bacterium would be categorised as non-differentiating.

1.2.2 Genetics of *H. pylori*

The genome sequence of *H. pylori* 26695 was released by The Institute for Genomic Research (TIGR) in June, 1997, and the annotated version was later published by Tomb *et al* (1997) during the course of this thesis work. The genome is 1.67Mb in size and contains 1590 coding sequences, of which 1091 have been assigned putative identities. The G+C content of the genome is 39%, as opposed to 34-35% as determined by thermal denaturation (Béji *et al*, 1988), and there are five distinct G+C regions with an either higher or lower percentage, one of which is the *cag* pathogenicity island which will be discussed in below.

The genomic arrangement of *H. pylori* suggests that the basic mechanisms of replication, cell division, and protein secretion are similar to those of *E. coli* and *H. influenzae*, although some important features appear to be missing. A protein involved in delivering DnaB helicase to the oriC-type primosome formation, DnaC, appears to be missing, suggesting that *H. pylori* has a novel mechanism for recruiting DnaB or has an orthologue with no detectable sequence similarity (Tomb *et al*, 1997). Also of importance is the transcription of the bacterial chaperone genes. Although the bacterial chaperones of protein secretion are present, the

transcription must differ to that of other bacteria as *H. pylori* does not possess the heat shock (σ^{32}) or stationary-phase (σ^{s}) sigma factors.

The recombination, repair and restriction systems of *H. pylori* appear to be similar to those of other bacteria, although several genes appear to be absent from the genome such as those of the *RecBCD* pathway which mediate homologous recombination and double-strand break repair. The transcription and translation systems are also similar to those of *E. coli*, with the exception of the genes for a catalytic activity in tRNA maturation, which appear to be absent in *H. pylori*.

H. pylori strains show considerable genetic variation, both at a nucleotide level (Labigne, 1997; Majewski *et al*, 1988) and in the gene organisation (Jaing *et al*, 1996; Desal *et al*, 1993). The genetic variation at the nucleotide level has allowed typing of isolates. As base pair substitutions are common in the genome, restriction endonuclease recognition sites can be altered, producing a distinct 'fingerprint' in each isolate (Fujimoto *et al*, 1993; Majewski *et al*, 1988).

1.3 Pathology of gastric disease and pathogenesis of *H. pylori*

The pathogenic affect on the upper gastrointestinal tract by *H. pylori* is 'slow' as symptoms may take decades to appear (Blaser, 1993). There are many outcomes of *H. pylori* infection. Firstly, all infections result in chronic gastric inflammation but while the extent of inflammation may vary, the condition is usually asymptomatic (Blaser, 1990). Consequently, many cases go undetected. If symptoms do occur, they represent chronic superficial gastritis. Chronic

superficial gastritis caused by *H. pylori* is a type B gastritis which is usually limited to the antrum of the stomach (Blaser, 1990). The inflammation caused by *H. pylori* infection may affect gastric secretory function, resulting in hypochlorhydria (Blaser, 1992). In the past, type B gastritis was treated with antacid but recurrences were common. As *H. pylori* is now recognised as the causative agent of this type of gastritis, therapy now involves antimicrobial agents, resulting in a low percentage of recurrences (Hentschel *et al*, 1993).

In some patients, chronic infection can develop and ulcers are formed. This has, in the past, been attributed to high levels of stress and alcohol, and although *H. pylori* is now thought to be the primary cause of the disease, other factors do contribute to the development of ulcers. These factors include smoking, age, and genetic background. Gender is also a factor and it has been reported that males are afflicted by diseases associated with *H. pylori* more frequently than women (Replogle *et al*, 1995). In a fourth group of patients, glandular integrity may be lost possibly due to the inability of the immune system to downregulate inflammation (Blaser, 1993). This can lead to chronic atrophic gastritis, a well-known pre-cursor to gastric adenocarcinoma (Forman *et al*, 1991;Correa, 1992).

1.3.1 Virulence Factors

H. pylori inhabits the mucosal layer overlying the gastric epithelium cells. It does not colonise all areas of the stomach and will only overlay gastric-type epithelial cells. These may be present in the stomach or be metaplastic in the duodenum (Buck *et al*, 1986). As a result, several virulence factors are implicated in disease. Some of these are outlined below.

1.3.1.1 Urease activity

The urease enzyme is thought to be central to the pathogenesis of *H. pylori* infection. The enzyme converts urea to ammonia and carbon dioxide, the release of ammonia ions raising the pH of the stomach to a more neutral level, and thereby providing protection from exposure to acid (Hu and Mobley, 1990). However, the bacteria colonise the space underneath the mucous layer where the pH approaches neutrality. The urease is nethertherless produced in large quantities. It has been hypothesised that such large quantities of the enzyme may protect the cells during initial colonisation of the stomach, and also in the rare event of exposure to low pH where no time is available to up regulate the production of urease (Bauerfeind *et al*, 1997). This role of the urease enzyme may not be unique. A recently isolated and characterised enzyme, aliphatic amidase, was found to produce ammonia (Skouloubris *et al*, 1997), thus suggesting that the main role of urease may not be in local pH elevation, but in causing direct damage to host cells as described below.

The *H. pylori* urease enzyme is novel among documented bacterial ureases as it is composed of two subunits rather than the usual three subunits. The two subunits are encoded by *ureA* and *ureB*, producing proteins of 30kDA and 62kDA, respectively. *H. pylori* urease has been shown to be more active that any bacterial urease characterised to date (Mobley *et al*, 1988). The activity is twice that of *Proteus mirabilis*, and ten times that of any other urinary tract isolates. Urinary tract isolates have higher urease activity than other bacteria due to the presence of high concentrations of urea in their environment. The low Michaelis constant (K_m) of the enzyme permits the enzyme to be efficient even at low urea concentrations (Hu and Mobley, 1990; Dunn *et al*, 1990). Mutants lacking the urease enzyme are unable to effect

the change in pH and subsequently die during the colonisation phase (Blaser, 1993). This action, therefore, makes the *H. pylori* urease genes potential targets of therapeutic agents.

The urease enzyme has also been shown to play an active role in causing disease symptoms of *H. pylori* infection. The products of the breakdown of urea can directly cause cell injury, and may cause vacuolation of gastric cells (Barer *et al*, 1988; Smoot *et al*, 1990; Xu *et al*, 1990). The ammonium ion can become water equilibrated to form ammonium hydroxide, a toxic compound that causes histological damage (Suzuki *et al*, 1992). Urease is also able to interact with the immune system, activating monocytes, polymorphonuclear leukocytes, and inflammatory response cells. This leads to indirect damage to the gastric epithelium (Suzuki *et al*, 1992). A further means of causing damage to the cells is through urease or urease-containing fractions to act as chemotactic factors for leukocytes, causing further local inflammation (Craig *et al*, 1992).

1.3.1.2 Motility

An important aspect of *H. pylori* pathogenesis is initial colonisation of the gastric mucosa. The stomach has two major means of defence against bacterial invaders - high acidity and peristalsis. Peristalsis may be largely countered by the motility of the bacterium. In order to colonise the gastric mucosa, *H. pylori* must be able to move quickly to limit the amount of time spent in an acid environment and then penetrate the mucous layer covering the gastric epithelium. As such, the flagella of *H. pylori* are vital to colonisation. Mutants lacking the ability to synthesis flagella are unable to colonise the stomach and are probably shed more quickly from the stomach by peristalsis (Eaton *et al*, 1992).

1.3.1.3 Cytotoxins

The product of the cytotoxin associated gene (CagA) and the vacuolating cytotoxin (VacA) are virulence factors that are expressed in a subset of clinical isolates (Xiang *et al*, 1995). While all strains carry the gene for VacA, it is only secreted in half of these strains (Cover *et al*, 1990). The *cagA* gene, however, is present in only some strains. This will be discussed below. Strains that lack *cagA* and do not secrete VacA may still cause damage to the gastric mucosa through the action of the urease enzyme, as described above.

1.3.1.4 Pathogenicity Islands

cagA, unlike *vacA*, is not present in all strains and those that do possess it, appear more virulent, causing more severe symptoms. *H. pylori* strains may be divided into two categories - those that cause disease with severe symptoms (type I) and those that cause disease with more mild symptoms (type II). A region of DNA in the chromosome of *H. pylori* has been shown to be present in the former strains. This region has been identified as the pathogenicity island (PAI) of *H. pylori* (Censini *et al*, 1996). Strains possessing this PAI are more frequently isolated from patients presenting serious clinical manifestations of duodenal ulcers, that is, type I strains contain the PAI (Hacker *et al*, 1997).

The PAI is approximately 40kb in length and appears to contain *cagA* and several other homologues encoding proteins involved in various secretory pathways (Censini *et al*, 1996). One of the predicted gene products has been provisionally named 'Pic' or 'promotes induction of cytokines' (Tummuru *et al*, 1995). Also present in the PAI are Vir and Tra proteins,

involved in the transfer of DNA, and Ptl proteins, involved in toxin export. The PAI is linked to the glutamate racemase gene (*glr*) and is flanked by 31bp direct repeats. The G+C content of the PAI differs to that of the rest of the genome, being 35% compared to 38-45% for the rest of the genome (Censini *et al*, 1997). This implies that the PAI may have been acquired from another source during evolution of *H. pylori*.

While some strains may possess the PAI, the virulence of the strains may be described as intermediate. This may be due to the presence of the insertion sequence IS605 which interrupts the PAI. The type strain, *H. pylori* CCUG 17874, contains such a PAI (Censini *et al*, 1996).

1.3.1.5 Glutamine Uptake

Stark *et al* (1997) have recently suggested that utilisation of glutamine by *H. pylori* may also be an indirect virulence factor. Glutamine has been shown to be deaminated by *H. pylori* to produce ammonia and glutamate through the action of glutamine deaminase. Glutamine is also the major amino acid present in serum, being the major nitrogen carrier in the human body. It is essential for the health of the epithelial cells of the small intestine and it is the primary energy source for lymphocytes. Uptake of glutamine by *H. pylori*, therefore, may have a direct inhibitory effect on the immune system leading to persistence of colonisation rather than eradication.

1.4 Epidemiology of *H. pylori*

It has been estimated that 53% of the world's population is infected with *H. pylori*, although most of these infections are asymptomatic (Graham *et al*, 1988). The incidence of infection differs between developing and developed countries. In developing countries, the incidence is approximately 70-90% with almost all infections occurring before the age of ten. In developed countries, the incidence is lower, ranging from 25-50%, although these infections are still mostly acquired during childhood. The statistics demonstrate a decline in the number of cases of *H. pylori* infection in developed countries with less than 10% of children now becoming infected (Fiedorek *et al*, 1991; Vandenplas *et al*, 1992). Prevalence is also associated with age, such that approximately 20% of twenty year olds will be infected, with a 1% increase with each subsequent year (Graham *et al*, 1988; Lindkvist *et al*, 1996). The prevalence is also higher in families with young children, in overcrowded homes where children share beds, and in families with no fixed hot water source (Mendall and Northfield, 1995). It is now believed that infection rates in children are the main determinant of population prevalence (Neale and Logan, 1995) while infection of adults is relatively uncommon with 0.3-0.5% per year in developed communities (Parsonnet *et al*, 1992; Kuipers *et al*, 1993). As the infection is 'slow', taking decades for symptoms to appear, the most important age in which the infection is acquired is in childhood (Lindkvist *et al*, 1996). Infection at this age generally results in symptoms appearing during middle age.

Socio-economic status also affects the incidence of infection. Families from a lower socio-economic group generally have a higher rate of infection than those from a middle to high group (Neale and Logan, 1995). Fraser *et al* (1996) conducted a study of school children

from South Auckland and demonstrated that Maori and Pacific Island children have higher rates of seropositivity for *H. pylori* than New Zealand European children from a similar socio-economic group. These results suggest that each ethnic group may have unique risk factors, but these have yet to be defined. Campbell *et al* (1997) have shown that ethnic groups are predisposed to infection by certain strains of *H. pylori* but the risk factors are still, as yet, undefined.

1.5 Transmission of *H. pylori*

1.5.1 Animal reservoirs and vectors of *H. pylori*

Few animal reservoirs exist for *H. pylori* and it is still not known how these reservoirs contribute to human disease (Fox, 1995). *H. pylori* strains have been isolated from cats, dogs, and some non-human primates. However, these reservoirs may only be important in regards to those that are in close contact with animals (Fox, 1995).

Houseflies have been shown to be a vector for transmission of *H. pylori*, carrying the bacteria on the body and in the digestive system of the insect (Grubel, 1997). Viable *H. pylori* cells have been isolated from fly excrete, indicating that the bacterium is able to replicate in the gut, or that the gut functions as a medium that preserves viability. As a result, the bacterium is able to be spread throughout the human environment, allowing higher probability of transmission. Houseflies may facilitate the transmission of *H. pylori* in all three of the routes of transmission described below.

1.5.2 Routes of transmission of *H. pylori*

The transmission of *H. pylori* is still not fully understood. The faecal-oral and the oral-oral route are the suspected routes of transmission but evidence exists for and against each. Other routes have also been suggested such as the gastro-gastric route and the gastro-oral route. The current status of our understanding of these routes is discussed below.

1.5.2.1 The faecal-oral route

H. pylori DNA has been detected by PCR and even isolated from stools of patients with gastritis (Thomas *et al*, 1992; Kelly *et al*, 1994). Furata *et al* (1994), however, studied the seroprevalence of *H. pylori* and compared it to that of hepatitis A virus (HAV) which is a marker of faecal-oral exposure. It was concluded that the transmission routes of *H. pylori* are independent to those of HAV and, therefore, the faecal-oral route is of limited relevance in a developed country. Further evidence against the faecal-oral route for transmission was provided by Marshall *et al* (1985) and Morris *et al* (1987). Two volunteers took oral cultures of *H. pylori* in an attempt to cause infection. Infection was achieved only with difficulty and the infectious doses were found to be significantly higher than the number of *H. pylori* present in faecal material, making it unlikely that this is the common means of transmission.

1.5.2.2 The oral-oral route

The rational behind the oral-oral route of transmission is that *H. pylori* can reach the oral cavity via regurgitation (Mégraud, 1995). Evidence for an oral-oral route of transmission, like that for faecal-oral transmission, is contradictory. Attempts to isolate or detect *H. pylori* from

the mouth have been generally unsuccessful although some researchers have reported the presence of *H. pylori* in dental plaque. Krajden *et al* (1989) detected *H. pylori* by PCR in one patient out of 29 while Cammarota *et al* (1996) detected *H. pylori* from one patient out of thirty one. *H. pylori* has also been cultured from the saliva of one out of nine patients known to carry the bacterium in the stomach (Ferguson *et al*, 1993). In both the Krajden *et al* (1989) and the Ferguson *et al* (1996) studies, the dental plaque and saliva strains were found to be identical to those present in the stomach of the patients, thus supporting the theory of gastric reflux.

1.5.2.3 The gastro-gastric and gastro-oral routes

Evidence has been well documented for the transmission of *H. pylori* via inadequately disinfected gastric endoscopy equipment (Langenberg *et al*, 1990; Sugiyama *et al*, 1992). Transmission by direct gastro-gastric contact occurs with ease, contrasting to faecal-oral and oral-oral route transmission. This has lead to a new hypothesis regarding the transmission of *H. pylori*, through a gastro-oral route spread by vomitus (Axon, 1995). During initial infection of children, an acute disease may occur which results in symptoms that are commonly seen in childhood - epigastric pain, flatulence, and vomiting. At this time acid secretion is prevented in the stomach and as a result, the vomit is mucousy and lacking in acid. This would provide a more suitable vector for transmission, as the lack of acid would enable *H. pylori* to survive for a greater length of time. A mucousy vomit is hard to clean, particularly if there is no fixed hot water source and, as such, the bacterium could be spread to other children with ease. This suggested transmission route fits well with the epidemiological data regarding infection during childhood.

In summary, *H. pylori* must have an effective transmission route for it to be able to colonise nearly half the world's population, but it is still not clearly understood. It may be that all these routes may coexist rather than one predominating (Mégraud, 1995).

Recently, a high incidence of paediatric gastritis was reported in Peru (Hulten *et al*, 1996). The drinking water was subsequently tested and *H. pylori* DNA was detected by PCR. This association between the presence of *H. pylori* in the water supply and the increase in gastritis in the local area implies that *H. pylori* may be able to survive in conditions where the carbon source is limited. This is supported by the work of Shahamat *et al* (1993) in which *H. pylori* was suspended in river water or undistilled water, and incubated at different temperatures. It was found that at 4°C the viable cell count decreased from 10^8 to 0 colony forming units (cfu) per ml between 20 and 25 days. In comparison, a liquid culture at 37°C will register no viable cell counts after 4-5 days (Reynolds and Penn, 1994). The ability of *H. pylori* to survive in an environment lacking in carbon is thought to be due to the carbon starvation response.

1.6 The carbon starvation response

The carbon starvation response may be defined as the systematic synthesis of proteins involved in the escape of starvation or in the development of a resistant state in the absence of a carbon source. It not well understood at present but has been studied in several bacteria. These include differentiating bacteria such as *Bacillus* and *Clostridia* sp., and non-differentiating bacteria such as *E. coli* and marine *Vibrio* sp. There is still debate as to which category *H. pylori* should be placed into due to the existence of the possibly dormant coccoid form. *H. pylori*, however, has no mechanism for endospore formation (Tomb *et al*, 1997) and since

coccoid, viable but non-culturable cells have been observed in other non-differentiating bacteria (Oliver, 1993), the carbon starvation response of *H. pylori* is thought to be similar to that of non-differentiating bacteria.

Relatively little is known about the carbon starvation response in non-differentiating bacteria. Study of marine *Vibrio* sp. has shown that the starvation response includes a time dependent series of molecular and physiological rearrangements accompanied by the sequential synthesis of the carbon starvation proteins. It has also been found that those proteins synthesised early in the response are the most important for survival (Nystrom *et al*, 1992).

1.6.1 The carbon starvation response of *E. coli*

Bacterial starvation responses can be induced by depletion of a number of nutrients. Phosphorus, nitrogen, and carbon depletion result in different starvation responses (Matin, 1991). Common to all three of these responses are a set of proteins involved in the heat shock response which is induced by a variety of cellular stresses such as starvation, osmotic, and oxidative stress (Matin, 1991). Included in this set of proteins are the chaperones DnaK, GroEL, and HtpG which are thought to accompany unfolded proteins across the membrane. DnaK also has proteolytic activity that makes it necessary in the carbon starvation response, allowing general protein turnover, or by degrading proteins that could have a deleterious effect if present during starvation.

The carbon starvation response is made up of three phases - a stringent response in an attempt to escape starvation, a relaxed phase, and then a phase involving the development of a

resistant state. The stringent response is a regulatory network with pleiotropic effects on cellular physiology that is specific to the depletion of individual nutrients (Flardh *et al*, 1994). The carbon starvation stringent response is induced by the exhaustion of amino acids or glucose, activating the *relA* gene (ppGpp synthetase I) or the *spoT* gene (ppGpp synthetase II), respectively (Ostling *et al*, 1996, Nystrom *et al*, 1994). RelA and SpoT produce 3',5'-bispyrophosphate (ppGpp), a well documented bacterial global regulator (Herzer, 1996). SpoT also has a ppGpp degrading activity. An increase in intracellular ppGpp leads to the induction of various genes involved in carbon starvation and has also been shown to have a role in the phosphotransferase pathway, a signal transduction pathway that results in the conversion of AMP to cAMP (Crasnier, 1996). cAMP is, in turn, able to induce a subsequent set of carbon starvation genes which belong to the *cst* group of genes (Blum *et al*, 1990; Matin, 1991).

Study of the carbon starvation response in *E. coli* has shown that more than fifty genes are involved which can be separated into two groups. The first group is comprised of the *cst* genes whose products are implicated with the escape from starvation by enhancing the cellular metabolic potential rather than the development of a resistant state (Blum *et al*, 1990). *cst* genes are involved in uptake and catabolic functions, and most are regulated by cAMP (Matin, 1991). The second group is the *pex* genes, which are involved with the development of a resistant state rather than the escape from starvation, a response that is more specialised as it involves physical resistance. The *pex* genes are those that are common to all starvation responses and included many heat shock proteins such as the 'chaperone' genes, *dnaK*, *groEL*, and *htpG*. Regulation of the *pex* genes is mostly by secondary sigma factors such as σ^{32} and σ^{38} (Matin, 1991).

1.6.2 CstA

The most well documented *cst* gene of *E. coli* is *cstA* (also referred to as *cst-2*) (Schultz and Matin, 1991). It is now known that the gene is directly induced by cAMP (Blum *et al*, 1990) and promoter mapping has revealed a -10 consensus sequence for the general sigma factor, σ^{70}. The -35 consensus sequence is not present but this is not uncommon for cAMP regulated promoters (Busby and Buc, 1987). The CstA protein has multiple hydrophobic domains which suggest that the protein is membrane associated (Schultz and Matin, 1991). The function of CstA has been studied by Schultz and Matin (1991) but an exact role was not determined. A *cstA*$^-$ mutant was compared to the wild type strain in growth, ability to survive carbon starvation, ability to metabolise various carbon sources, and the bulk protein degradation rate. No differences were observed. The involvement of cAMP, known to control uptake systems, and the location of the protein in the membrane lead to the consideration of a role in transport. The dipeptide permease (*dpp*) genes had been roughly mapped to the *cstA* locus in *E. coli* so peptide transport, in particular, was studied. The major peptide transport locus (*opp*) was knocked out and only *cstA opp* double mutants demonstrated impaired growth on a tryptone based medium. It was concluded that CstA has a role in increasing the range of substrates that the cell could utilise to escape starvation.

1.6.3 The carbon starvation response of *H. pylori*

1.6.3.1 Metabolism of H. pylori

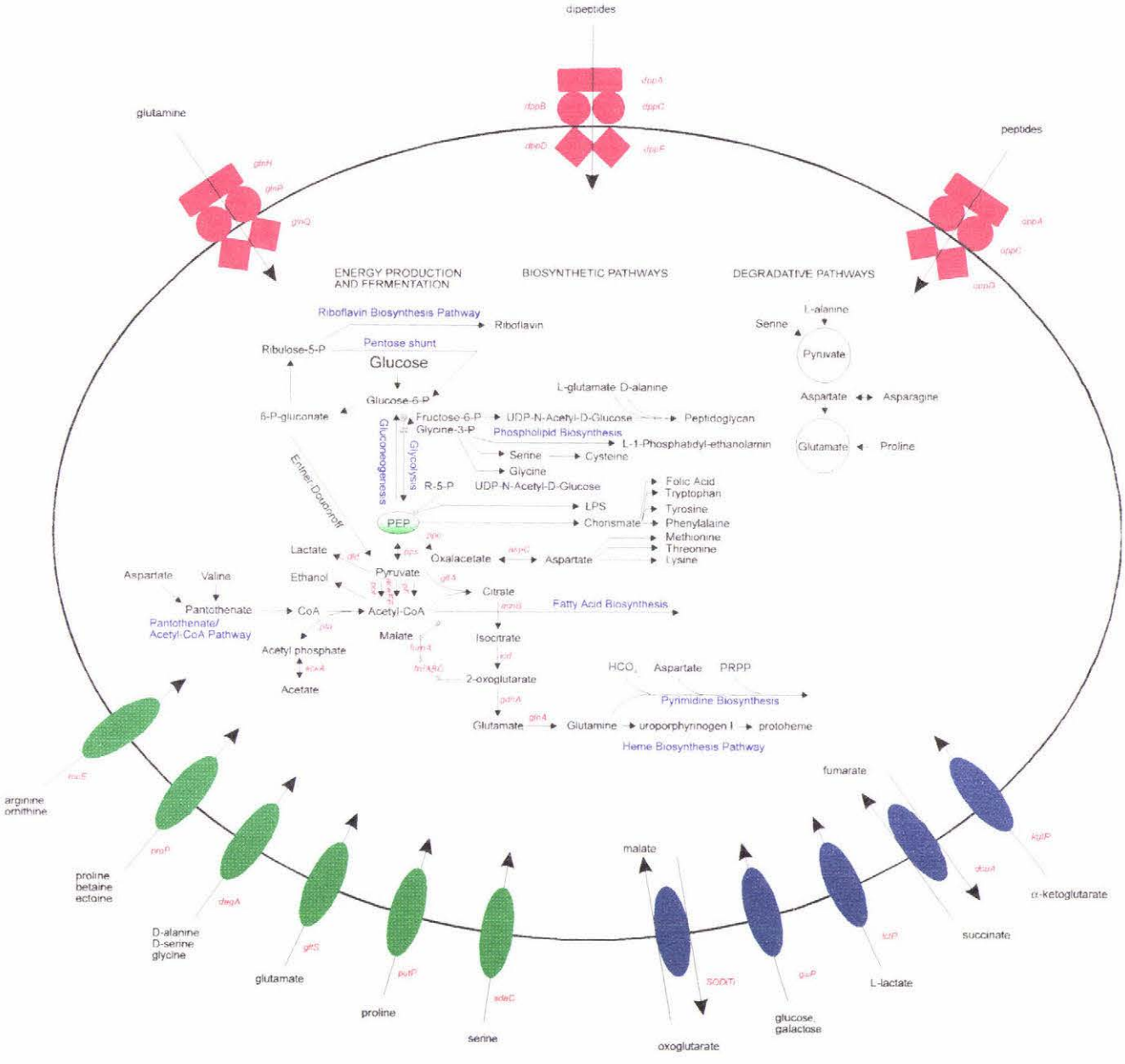
An important factor in the carbon starvation response of *H. pylori* is identifying the carbon and energy sources. Of relevance to this thesis work, analysis of the *H. pylori* genome has

facilitated prediction of several metabolic pathways (Tomb *et al*, 1997) (Fig1.1). Firstly, *H. pylori* uses glucose as the only source of carbohydrate and also derives energy from the degradation of serine, alanine, aspartate, and proline. Secondly, several substrates can be used as a source of nitrogen, including urea, ammonia, alanine, serine, and glutamine. The use of glutamine as a nitrogen source is further supported by the presence of a consensus sequence for the sigma factor associated with nitrogen starvation, σ^{54}, in the promoter region of the *glnH* homologue.

It is now well established that *H. pylori* is able to utilise amino acids as a sole carbon source (Nedenskov, 1994; Stark *et al*, 1997). Reynolds and Penn (1994) have also studied the utilisation of amino acids and, although it was concluded that eight amino acids were required for growth, it was also concluded that amino acids could not be used as a sole carbon source. This is most likely due to the method in which amino acid utilisation was determined. Reynolds and Penn (1994) removed glucose from their medium and lowered all but the amino acid being tested to 20% of the documented concentration. This meant that the required amino acids that were not being tested were present in too low a concentration to promote growth, which subsequently concluded to be due to the lack of glucose. However, the results of the Reynolds and Penn (1994) and the Nedenskov (1994) studies showed that alanine, arginine, histidine, isoleucine, leucine, methionine, phenylalanine, and valine were required for growth. Nedenskov (1994) also showed that in some strains of *H. pylori,* proline was also required. The requirement for amino acids was determined by incubating the bacteria in defined medium lacking in individual amino acids. Stark *et al* (1997) used continuous culture techniques and tested the medium after a period of time for the utilisation of amino acids. Eight amino acids were found to be utilised during growth and these differed from those identified by Nedenskov (1994) and Reynolds and Penn (1994). Stark *et al* (1997)

Figure 1.1 Metabolic pathways and transport of substrates in *H. pylori*

Pathways for amino acid uptake, biosynthesis, and use and glucose metabolism are shown in this figure. Transporters are depicted in the surrounding ring, representing the cell membrane. Three groups of substrate transporters are depicted - blue ovals represent amino acids permeases, green ovals represent permeases of compounds involved in glucose metabolism, and red complexes represent ATP-binding cassette transporters. Source: Tomb *et al* (1997).


dipeptides
dppA
dppB
dppC
dppD
dppE
glutamine
glnH
glnP
glnQ
peptides
oppA
oppC
oppD
ENERGY PRODUCTION AND FERMENTATION
BIOSYNTHETIC PATHWAYS
DEGRADATIVE PATHWAYS
Riboflavin Biosynthesis Pathway
Riboflavin
Pentose shunt
Ribulose-5-P
Glucose
Glucose-6-P
6-P-gluconate
Fructose-6-P
UDP-N-Acetyl-D-Glucose
Peptidoglycan
Glycine-3-P
Phospholipid Biosynthesis
L-1-Phosphatidyl-ethanolamin
Serine
Cysteine
Glycine
Gluconeogenesis
Glycolysis
R-5-P
UDP-N-Acetyl-D-Glucose
Entner-Doudoroff
PEP
LPS
Chorismate
Folic Acid
Tryptophan
Tyrosine
Phenylalaine
Methionine
Threonine
Lysine
Lactate
Oxalacetate
Aspartate
Pyruvate
Ethanol
Citrate
Aspartate
Valine
Pantothenate
CoA
Acetyl-CoA
Fatty Acid Biosynthesis
Pantothenate/ Acetyl-CoA Pathway
Malate
Isocitrate
Acetyl phosphate
Acetate
2-oxoglutarate
HCO3
Aspartate
PRPP
Pyrimidine Biosynthesis
Glutamate
Glutamine
uroporphyrinogen I
protoheme
Heme Biosynthesis Pathway
L-alanine
Serine
Pyruvate
Aspartate
Asparagine
Glutamate
Proline
arginine
ornithine
proline
betaine
ectoine
D-alanine
D-serine
glycine
glutamate
proline
serine
malate
oxoglutarate
fumarate
succinate
L-lactate
glucose,
galactose
α-ketoglutarate

demonstrated that alanine, arginine, asparagine, aspartate, glutamine, glutamate, proline and serine were utilised by *H. pylori*. It is still unknown what the nutritional significance of each of these amino acids is in terms of the growth of *H. pylori*, although the analysis of Tomb *et al* (1997) has provided a platform for further research.

1.6.3.2 Carbon starvation genes of H. pylori

Several genes were identified in the *H. pylori* genome which are of importance to the carbon starvation response. A *spoT* homologue was found in the genome but a *relA* homologue, induced by amino acid starvation (Ostling *et al*, 1996), was absent. Studies with *relA*-mutants in *E. coli* and marine *Vibrio* sp. have demonstrated that the carbon starvation response is unaffected as the cells are able to accumulate the same levels of ppGpp through the action of *spoT* (Ostling *et al*, 1996).

Homologues of the *pex* gene group were also found to be present in the *H. pylori* genome sequence. These genes include the 'chaperone' genes *dnaK*, *groEL*, and *htpG*. In *E. coli*, these genes are regulated by the stationary-phase sigma factor (σ^{38}) or the heat shock sigma factor (σ^{32}). As previously discussed, the genes for these sigma factors were undetected in the *H. pylori* genome sequence, so it is likely that *H. pylori* has a different means of transcriptional regulation of some *pex* homologues than that of *E. coli* (Tomb *et al*, 1997).

The genome sequence was released during the course of this thesis work (see also Results and Discussion). However, some of the sequence information of submitted to GenBank prior to the availability of the genome sequence. The relevant features of *cstA* of *H. pylori* 26695 are

largely presented as follows. *cstA* was found to have 72% residue similarity with the *E. coli cstA*, and to possess 17 membrane spanning domains (Tomb *et al*, 1997). Additionally, a signal sequence of ten amino acid residues precedes the *cstA* sequence. This provides further evidence to Schultz and Matin's (1991) hypothesis that *cstA* was membrane associated. The evidence provided by Tomb *et al* (1997) suggests that the CstA homologue is located in the inner membrane.

1.7 Preliminary information

A plasmid, pHP040 (Appendix A.1), has been constructed by Dr Paul O'Toole carrying genes relevant to metabolism of *H. pylori*. Preliminary sequencing was carried out before the release of the genome sequence and identified a *cstA* homologue which was named the *c*arbon *s*tarvation *g*ene (*csg*), a permease that was shown to be an *E. coli glnP* homologue, and an ATP binding cassette that was shown to be an *E. coli glnQ* homologue. A gene was identified between the *H. pylori glnP* and *glnQ* but never sequenced. However, upon the release of the genome, it was discovered that this gene is a second *glnP* homologue. Due to the presence of two *glnP* homologues, the genes were named *glnP1* and *glnP2*. *glnP* is the glutamine permease gene, while *glnQ* is the glutamine permease ATP binding cassette. It was further discovered that the gene downstream of *glnQ*, which was not carried on pHP040, was a *glnH* homologue, which encodes for the glutamine permease periplasmic protein. *glnP*, *glnQ*, and *glnH* make up the glutamine permease operon (Nohno *et al*, 1986; Claverie-Martin and Magasanik, 1992). Protein identities and similarities for the above genes are shown in Table 1.1

Table 1.1 Percentage Identity and Similarity for genes of the *csg* locus

H. pylori Gene	Homologue[a] of	% Similarity	% Identity
csg	*E. coli cstA*	72	58
glnP1	*E. coli glnP*	56	30
glnP2	*E. coli glnP*	48	30
glnQ	*E. coli glnQ*	71	51
glnH	*E. coli glnH*	59	40
glnP1	*H. pylori glnP2*	44	30

[a] The homolgue listed for each *H. pylori* gene is that listed in BLAST results as having the highest p value.

1.8 Research Objectives

1.8.1 To investigate the role of the carbon starvation gene during the carbon starvation response.

To understand how *csg* might function during the carbon starvation response, it would first be necessary to sequence the gene to provide primary information such as the putative sigma factor involved in transcription and the possible location of the gene within the cell. The second part of this objective was to construct an *H. pylori* strain with a non-functional *csg* gene. In doing this, it would be possible to study the effect of the mutation during carbon starvation and to therefore elucidate the role of the gene during the response. The role of the glutamine permease operon would also be studied to a lesser extent to investigate its role during carbon starvation.

1.8.2 To investigate the regulation of *csg* with regards to the environment.

The second objective of this thesis project was to study the regulation of *csg* under various environmental conditions such as a change in cAMP, change in pH, and during osmotic stress. This would be achieved by quantifying the amount of *csg* transcripts, through the use of RNA analysis or by fusing a reporter gene to *csg*.

2.0 Methods and Materials

2.1 Bacterial strains

Bacterial strains used in this study are listed in Table 2.1

2.2 Media

2.2.1 Luria Broth (LB) - 10g/l tryptone; 10g/l NaCl; 5g/l yeast extract. The pH was adjusted to 7.5 prior to autoclaving by the addition of NaOH to give a concentration of 2.5mM NaOH. For solid medium, agar was added to 1.5%

2.2.2 Brain Heart Infusion (BHI) Broth for *H. pylori* - 37g/l brain heart infusion (BHI) medium ; 5% Fetal Calf Serum.

2.2.3 Defined Medium for *H. pylori* - The defined medium for *H. pylori* was made up as described by Reynolds and Penn (1994).

2.2.4 Chocolate Blood Agar (CBA) - 39g/l Columbia Base Agar (Oxoid); 5% Defibrinated Horse Blood. The medium was autoclaved before the addition of blood. It was then cooled to 50-70°C before the blood was added to avoid clotting. The medium was then heated until a brown/chocolate colour formed, cooled to 50°C and then poured in sterile plastic petrie dishes.

Table 2.1 Bacterial Strains and Plasmids

Bacterial Culture or Plasmid	Characteristics	Reference or Source
E. coli		
ER2206	endA1 thi-1 supE44 mcr67(mcrA) ∂(mcrBC-hsdRMS-mrr) 114::1S10 ∂(lac)U169/F' proAB lacI^q Z ∂M15 Tn10 (Tet^R)	NEB, (personal communication)
XL-1	SupE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 lac$^-$ F'[proAB$^+$ lacI^q Z ∂M15 Tn10 (Tet^R)	Stratagene Cloning Systems Catalogue (1994)
HB101	F$^-$ mcrBC mrr hsdS20(r$_B^-$, m$_B^-$) recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20(Sm^R) xyl5 ŧleu mtl1	Sambrook *et al* (1989)
H. mustelae		
4298	Ferrit isolate	Donated by J.G. Fox (Division of Comparative Medicine, Massachusetts Institute of Technology, Cambrigde, MA)
H. pylori		
CCUG 17874	Same as NCTC 11637, the *H. pylori* type strain.	Culture Collection of the University of Göteborg
Sydney strain	Infects both mice and humans	Lee *et al* (1997)
MU18	Clinical Isolate	Cambell *et al* (1997)
MU55	Clinical Isolate	Cambell *et al* (1997)
MU57	Clinical Isolate	Cambell *et al* (1997)
MU61	Clinical Isolate	Cambell *et al* (1997)
MU62	Clinical Isolate	Cambell *et al* (1997)
MU68	Clinical Isolate	Cambell *et al* (1997)
MU82	Clinical Isolate	Cambell *et al* (1997)
MU91	Clinical Isolate	Cambell *et al* (1997)
MU101	Clinical Isolate	Cambell *et al* (1997)
ΔglnP1	CCUG 17874 with a non-functional *glnP1* gene	P.W. O'Toole
Δcsg	CCUG 17874 with non-functional *csg* and *glnP1* genes	this study
SS Δcsg	Sydney strain of *H. pylori* with non-functional *csg* and *glnP1* genes	this study
csg::CAT	promoterless CAT cassette downstream of *csg*, Kn^R	this study
Plasmids		
pHP040	3.2kb *H. pylori* genomic insert in pBK-CMV	P.W. O'Toole
PHP041	Based on pHP040 with Kan^R from pRY109 cloned inot the *EcoR*V site	P.W. O'Toole
pHP042	3.4kb *H. pylori* genomic insert with attached 163bp *Hind*III fragment in pBK-CMV	P.W. O'Toole
pAB01	2.6kb PCR fragment containing 3' terminus of *csg*	this study
pAB02	Insert of pHP040 lacking 300bp region containing *csg* and *glnP1* promoter , replaced with a *Bgl*II site, and cloned into pUC19	this study
pAB03	KanΩ cloned into *Bgl*II site of pAB02	this study
pAB04	*Bgl*II site 10bp downstream of *csg* in pAB01	this study
pAB05	CAT and Kan^R cassettes cloned into the *Bgl*II site of pAB04	this study
pILL600	Kn^R with flanking symmetrical multiple cloning sites	Labigne *et al* (1988)
pCM4	Promoterless CAT with flanking *Bam*HI sites	Close and Rodriguez (1982)
pUC19	Amp^R LacZ'	Yanisch-Peron *et al* (1985)
pUC4omega-km2	Kn^R with flanking Ω regions	Perez-Casal *et al* (1991)

2.2.5 Tryptone Soya Broth (TSB) - 30g/l Tryptone Soya Broth (Oxoid).

2.2.5 Semi-defined medium for *H. pylori* - The semi-defined medium was based on that of Reynolds and Penn (1994). All amino acids were replaced with tryptone (17g/l).

2.3 Growth conditions

E. coli was grown over night on solid medium in an aerobic laboratory incubator at 37°C. All liquid culture *E. coli* strains were grown over night in a lab shaker at 37°C, shaken at 190 rpm. *H. pylori* strains grown on solid medium were grown in a controlled atmosphere laboratory incubator under microaerophilc conditions with 5% CO_2 over a period of 3 days. *H. pylori* strains grown in liquid cultures were incubated for approximately five days in an anaerobic jar with a CO_2 emitting gas pack (Oxoid). The anaerobic jar was placed in the laboratory shaker and shaken at 190rpm.

2.4 Common buffers and solutions

2.4.1 1 x TAE 40mM Tris; 19mM Glacial Acetic Acid; 1mM EDTA

2.4.2 TE buffer 10mM Tris; 1mM EDTA

2.4.3 Tris/HCl 1M Tris; concentrated HCl was added dropwise to pH the solution. Solutions were made at pH 7.5, 8.0, and 9.5

2.4.4 EDTA - 0.5M Ethylenediaminetetra-acetic acid disodium salt (EDTA)

2.4.5 Cell Lysis Buffer - 0.2M NaOH; 1% SDS

2.4.6 Neutralising Buffer 1 - 20% sodium acetate

2.4.7 Neutralising buffer 2 - 1.32M potassium acetate, pH 4.8

2.4.8 Loading Buffer - 0.05g Bromophenol Blue; 0.05M EDTA; 50% glycerol; made up to 10ml with 1 x TAE

2.4.9 Phosphate Buffered Saline - pH 6.8 - 24.5ml 0.2M Na_2HPO_4, 25.5ml 0.2M NaH_2PO_4

2.4.10 Urease Testing Solution - 0.01M PBS pH 6.8; 0.001% Phenol Red; 2% (w/v) urea

2.4.11 Dig Buffer P1 - 0.1M Maleic acid; 0.15M NaCl

2.4.12 Dig Buffer P2 - Dig buffer P1 + 1% blocking reagent

2.4.13 Dig Buffer P3 - 0.1M Tris/HCL pH 9.5; 0.1M NaCl; 0.05M $MgCl_2$

2.4.14 Dig Hybridisation Solution - 5 x SSC; 1% blocking reagent; 0.1% N-laurylsarkosin; 0.02%SDS

2.4.15 20 x SSC - 3M NaCl; 0.3M Na-citrate; pH 7

2.4.16 GES - 5M Guanidium Thiocynate; 100mM EDTA; 0.5% (w:v) Sarkosyl

2.4.17 Easy Prep Lysis Buffer - 0.01M Tris; 1mM EDTA; 3g sucrose; 40mg lysozyme; 400µl RNAse (10mg/ml); 2mg BSA; made up to 20ml with SDW

2.4.18 50 x MOPS EA - 1M MOPS; 0.25M sodium acetate; 50mM EDTA

2.5 DNA preparations

2.5.1 Alkaline lysis plasmid preparation with polyethylene glycol (PEG) precipitation

This preparation consists of an alkaline lysis stage followed by PEG (Polyethylene glycol) precipitation. An overnight bacterial culture was centrifuged in a microfuge for 20 seconds to pellet the cells. The supernatant was discarded and the pellet then resuspended in 250µl of TE (Section 2.4.2). Vortexing or pipetting was used to break up the pellet. To this suspension, 250µl of cell lysis buffer (Section 2.4.5) was added and the solution mixed by gentle inversion. The solution was left at room temperature for 10 minutes before 250µl neutralisation buffer 1 (Section 2.4.6) was added. The solution was mixed by gentle inversion, then centrifuged at 14,000rpm for 15 minutes. The supernatant was then removed to a fresh microfuge tube. 2µl of RNAse (10mg/ml) was added to the supernatant and incubated at 37°C for 20 minutes. An equal volume of chloroform was then added to the solution, mixed by vortexing, and

centrifuged at 14,000 revolutions per minute (rpm) for 5 minutes. The top layer was removed to a fresh microfuge tube and an equal volume of isopropanol was added to the solution. The solution was mixed and centrifuged at 14,000rpm for 15 minutes. The supernatant was discarded, the pellet washed with 200µl of 70% ethanol, and then left to air dry. The dry pellet was resuspended in 32µl of sterile distilled water (SDW). To this was added 8µl of 4M sodium chloride and 40µl of 15% PEG. The solution was vortexed and left on ice for at least 30 minutes. The solution was then centrifuged at 14,000rpm for 15 minutes, the pellet washed with 200µl of 70% ethanol, and left to air dry. Once dry, the pellet was resuspended in 30µl of SDW.

2.5.2 Quick alkaline lysis plasmid preparation

This plasmid preparation was a quick method for screening colonies from a transformation for the desired size. The preparation is crude but the resulting DNA can be digested to completion by restriction enzymes. 2ml bacterial cultures were centrifuged for 30 seconds to pellet the cells. The supernatant was discarded and the pellet resuspended in 200µl of TE (Section 2.4.2). 2µl of RNAse (10mg/ml) was added and the solution mixed. 200µl of cell lysis buffer (Section 2.4.5) was added to the solution and mixed by gentle inversion. The solution cleared almost immediately. 200µl of Neutralising buffer 1 or 2 (Section 2.4.6 and 2.4.7) was then added to neutralise the solution which was then centrifuged at 14,000rpm for 10 minutes. The supernatant was removed to a fresh microfuge tube and an equal volume of chloroform was added. The solution was vortexed to emulsify the two layers and then centrifuged at 14,000rpm for 5 minutes. The upper layer was removed to another fresh microfuge tube and an equal volume of isopropanol was added. The solution was mixed

thoroughly and centrifuged at 14,000rpm for 10 minutes. The supernatant was removed completely and the pellet was left to air dry. The dry pellet was then resuspended in 50µl of SDW and 5µl was examined by gel electrophoresis (Section 2.6.3) if the culture was grown on the same day, or resuspended in 30µl and 1µl checked by gel electrophoresis if the culture was grown over night.

2.5.3 Large scale plasmid preparation

A starter culture was grown in 2ml of selective broth during the day. This was used to inoculate 500ml of selective broth, which was then grown up over night. The cells were harvested by centrifugation in a GSA rotor at 6000rpm at 4°C for 10 minutes. The supernatant was discarded and the pellet resuspended in 3ml of TE. The suspension was then transferred to SS34 tubes. 3ml of cell lysis buffer was added to the suspension, mixed by gentle inversion, and left to stand at room temperature for 10 minutes. 3ml of neutrilisation buffer 1 was then added to neutralise the solution, mixed by gentle inversion and left to stand at room temperature for 10 minutes. The solution was centrifuged at 15000rpm at 4°C for 20 minutes and the supernatant was decanted into a fresh SS34 tube. 20µl of RNAse (10mg/ml) was added to the solution and incubated at 37°C for 15 minutes. The solution was then transferred to falcon tubes and a chloroform extraction was performed by adding an equal volume of chloroform to the solution, vortexing for 30 seconds to mix, and then centrifuging for 30 seconds. The chloroform layer (the lower layer) was removed and the extraction repeated. The solution was then transferred back to SS34 tubes. An equal volume of isopropanol was added to precipitate the DNA and the solution was centrifuged at 10,000rpm at 4°C for 10 minutes. The supernatant was discarded and the pellet resuspended in 1ml TE.

The solution was transferred to a small falcon tube and the volume made up to 5.7ml with TE. 0.1ml of Ethidium Bromide (10mg/ml) was added to the solution, followed by exactly 6g of caesium chloride. The solution was carefully mixed to dissolve the caesium chloride and then transferred to a 6ml Sorvall Ultracentrifuge tube. The solution was centrifuged at 55000rpm for 18 hours. After ultracentrifugation, a plasmid band was visible below a chromosomal DNA band. The plasmid band was removed using a syringe, and transferred to a microfuge tube. An equal volume of caesium chloride saturated isopropanol was then added to the solution, vortexed for 30 seconds, and centrifuged at 14,000rpm for 30 seconds. The top layer was discarded. This was repeated until the solution became clear. The solution was then transferred to a length of dialysis tube that had been prepared by boiling for 5 minutes in water and letting cool. The caesium chloride DNA was dialysed against Milli-Q water for 4 hours at 4°C. After dialysis, the DNA was divided into 500µl aliquots. To each of these aliquots, 0.1 volume of 3M sodium acetate and 2.5 volumes of cold 100% ethanol were added. The microfuge tubes were left at -80°C for 30 minutes. The solutions were then centrifuged at 14,000rpm for 20 minutes, the pellets washed with 200µl 70% ethanol, and the pellet allowed to air dry. The dry pellets were resuspended in 200µl SDW and 1µl was examined by gel electrophoresis (Section 2.6.3).

2.5.4 Chromosomal quick preparation for Helicobacters

A heavily streaked plate was harvested by swabbing cells into 1.5ml of PBS (Section 2.4.8) in a microfuge tube. The tube was vortexed to break up any clumps and the solution pelleted by centrifugation at 14,000rpm for 20 seconds. The supernatant was discarded and the pellet resuspended in 100µl of TE by pipetting. 500µl of GES (Section 2.3.16) and 2µl and RNAse

(10mg/ml) was added to the solution and mixed by gentle inversion until the solution became clear. The solution was then left at room temperature for 10 minutes. 1ml of cold 95% ethanol was added to the solution to precipitate the DNA. The DNA was removed from the solution using a glass hook. It was then submerged in 70% ethanol and resuspended in 100-200µl TE. 20µl of proteinase K (50mg/ml) was added to the DNA solution and incubated at 50°C for 2-3 hours. After this time, protein associated with the DNA should have disappeared and the solution should have been clear. The DNA was recovered by adding 1ml of cold 95% ethanol and removing with a glass hook. It was submerged briefly in 70% ethanol and resuspended. If the DNA solution was still not clear, the DNA was resuspended in 100-200µl of TE, GES was added and the solution incubated as before. After recovering the DNA from a clear solution, the DNA was resuspended in 200-500µl of SDW, depending on the initial mass of cells. There does not appear to be any carry over of active proteinase K, and 1-5µl volumes of the DNA cut cleanly with various restriction enzymes.

2.5.5 Easy plasmid preparation

This procedure results in DNA which, due possibly to salt concentrations, does not migrate at the same rate as the molecular weight standard during gel electrophoresis. As such, it is used for confirming the presence of a plasmid rather than screening for a specific size of plasmid. This procedure is for *endA*$^-$ strains only. A 2ml bacterial culture was centrifuged at 14,000rpm for 30 seconds to pellet the cells. The supernatant was discarded, the pellet was resuspended in 30-100µl of Easy Prep Lysis Buffer (Section 2.3.17) and mixed by gentle inversion until clear. The solution was then shaken at room temperature for 5 minutes, boiled for 60 seconds, then chilled for 60 seconds. It was then centrifuged at 14,000rmp for 15-20 minutes and the

supernatant removed to a fresh microfuge tube. The plasmid DNA solution was then used without any further purification.

2.5.6 DNA purification by Phenol/Chloroform extraction and ethanol precipitation

An equal volume of Tris-equilibrated Phenol was added to the DNA solution and vortexed to emulsify. The solution was then centrifuged at 14,000rpm for 2 minutes. The upper layer was removed to a fresh microfuge tube. An equal volume of TE was then added to the phenol solution, vortexed, centrifuged for 2 minutes and the lop layer pooled with that of the first extraction. An equal volume of chloroform was then added to the solution, vortexed and centrifuged at 14,000rpm for 30 seconds. The lower chloroform layer was removed and the extraction was repeated. The DNA was precipitated by adding 0.1 volumes of 3M sodium acetate and 2.5 volumes of cold 95% ethanol to the solution. The solution was then left to stand at –80°C for 30 minutes. After this time, the solution was centrifuged at 14,000rpm for 15 minutes, the pellet was washed with 200µl of 70% ethanol, and the pellet left to air dry. Once dry, the pellet was resuspended in 10-50µl SDW.

2.6 Standard procedures for working with DNA

2.6.1 Restriction digests

Restriction digests were carried out in 30µl volumes with 0.2-1µg of DNA, 1-2 units of enzyme, and the appropriate restriction buffer. The volume was made up with SDW. Digests

were incubated at 37°C for 2-3 hours except for enzymes that required special incubation temperatures (ie: *Sma*I at 25°C).

2.6.2 Gel electrophoresis

DNA was size fractionated by electrophoresis through agarose gels of 0.8-1.2% at 80-100V. Gels were made up to volume with the running buffer, 1 x TAE (Section 2.3.1). Samples were mixed with 0.1 volume of loading buffer (Section 2.3.6). At the completion of electrophoresis, the gel was stained in an ethidium bromide solution for 10-20 minutes, rinsed in Milli-Q water, and observed under short wave ultraviolet light. The gel was photographed using Alphimage 2000 software.

2.6.2.1 Determining the molecular weight of DNA fragments

A set of standard markers were used to aid in estimating the molecular weight of DNA fragments. The standard was size fractionated concurrently with the sample. The standards used in this study were:

i. λ*Eco*RI/*Hind*III (Section2.6.2.2)

ii. 1kb ladder (Boehringer Manheim)

iii. 100bp ladder (Boehringer Manheim)

iv. covalently closed supercoiled (ccc) DNA ladder (BRL)

*2.6.2.2 Preparation of λEco*RI*/Hind*III *markers*

The λ*Eco*RI/*Hind*III standard was prepared by digesting 50ng of λDNA (New England Biolabs) with 100 units of *Hind*III and 100 units of *Eco*RI. The digest was performed in a 500µl volume and used the restriction buffer normally used with *Hind*III. The digest was incubated at 37°C for 2-3 hours after which time is was heated to 65°C for 15 minutes. 2µl was examined by gel electrophoresis. Working aliquots of 125µl were made with 50µl of digest, 12.5µl of loading buffer, and made to volume with TE.

2.6.3 Determination of DNA concentration

DNA concentration was determined by spectrophotometry. Light of wavelengths 260 and 280 was passed through suitably dilute DNA samples. The absorbance was measured and used to calculate the concentration of the DNA. An absorbance of 1.0 at A_{260} corresponds to a concentration of 50ng of double stranded DNA or 33ng of single stranded DNA. The purity of the DNA was indicated by the ratio of A_{260}/A_{280} where a ratio between 1.8-2.0 indicates pure DNA.

2.7 Polymerase Chain Reaction (PCR) amplification of DNA

DNA was amplified by means of the polymerase chain reaction (PCR) which exponentially amplifies targeted DNA. All PCR reactions performed in this study were carried out in 10µl volumes. A master mix of components common to all reactions was prepared while unique

components were added individually to each tube. Each reaction contained two oligonucleotide primers with a final concentration of 0.2μM each, dNTPS with a final concentration of 2.5μM, $MgCl_2$ with a final concentration of 2.5μM, and 10 x PCR buffer with a final concentration of 1 x PCR buffer. Primers used in PCR amplification of DNA are listed in Table 2.2. Each component concentration could be optimised by performing a titration of the component concerned. Approximately 200μg of DNA was added to each reaction, although this amount varied from template to template.

To each reaction, 0.5 units of DNA polymerase were added to carry out replication of the DNA. Two DNA polymerases were utilised in this study. *Taq* DNA polymerase was used on most occasions and produced a fragment with 5'-A overhangs. When a blunt ended fragment was required, *Pwo* DNA polymerase was used. It was also found that *Pwo* DNA polymerase was useful for low yielding reactions as it produced more DNA.

All reactions were carried out in 200μl NATURAL thin wall PCR tubes (Quality Scientific Plastics). Tubes were placed into a thermocycler (Corbett Research FTS-690 or Hybaid Omni-E) preheated to 92°C.

For the amplification of the region spanning the *glnP1* and *csg* transcription start points and the intergenic region, using primers CSG3 and C14, the following program was used. The annealing temperature was lower than would be expected based on the T_ms of the two primers. CSG3 has a T_m of 61°C and C14 has a T_m of 54°C., therefore, an annealing temperature of 52°C would be expected. However, attempts to perform the PCR amplification using this annealing temperature failed.

Table 2.2 PCR and Sequencing Primers

Primer Name	-mer	Tm (°C)[a]	Sequence (5' to 3')	Source
M13 (lacZ) forward primer	17	52	GTAAAACGTCGGCCAGT	Perkin Elmer
M13 (lacZ) reverse primer	20	55	GGAAACAGCTATGACCTTG	Perkin Elmer
CSG1	19	63	GCCCCAAGGCGAATGCGCT	P.W. O'Toole
CSG2	23	60	CCATCACCTTTGCGCATCATTTC	P.W. O'Toole
CSG3	23	61	GAAATGATGCCCAAAGGTGATGG	P.W. O'Toole
CSG4	22	53	CATAAACTTCCCATAATAAGGG	P.W. O'Toole
CSG5	17	55	ACGCGCTTTATCTCCCC	P.W. O'Toole
CSG6	18	49	CTTTATTTAACAGCTCCC	P.W. O'Toole
CSG7	18	49	CTGTTATTGGCTTTATCC	P.W. O'Toole
C8	18	56	ATGCCTTCTATAGGGGCG	P.W. O'Toole
C9	20	50	AAATCTAATGAGGCGGTTAC	P.W. O'Toole
C10	18	54	TTTGTAGATGGCAGTGGG	P.W. O'Toole
C11	19	52	AATGGAAGCCAAGCTCTTA	P.W. O'Toole
C12	20	55	CAGGGATGGAGTGAAACATA	P.W. O'Toole
C13	18	51	AAATGGAGAGGACATGCA	P.W. O'Toole
C14	18	54	GAGAGCTAAGAGCGATCA	P.W. O'Toole
C15	19	59	TATGGCGTTGGTGTGCGCA	P.W. O'Toole
C16	19	57	TTAGGGGTCGTTCCTGAAG	P.W. O'Toole
AB01	39	51	CCATCGTAAGATCTGGGCTGCGGTGTTAG CCTTACACAA	this study
AB02	43	55	GCAGCCCAGATCTTACGATGGGGTTAGAA CTCACGCTTTATAT	this study
AB03	21	64	GCGGGTTTTACAGGCACAGCC	this study
AB04	20	55	CCACTTTTGCGATCGGTTTA	this study
AB07	21	58	CAAAGAACGATGATAGCGTGC	this study
AB08	21	54	CCCTATTAAATTAACAGAGCC	this study
AB09	21	58	CACCACCGTAACCAACAACAA	this study
AB10	20	55	CGCCGATGTTTTTAGTCATC	this study
AB11	29	51	GAGAGATCTCCTTTTTTATTCAAGTGGCT	this study
AB12	30	51	AGGAGATCTCTCGGTTTTAACCCCCTTTAA	this study
CAT1	27	47	GGGAGATCTGAGATTTTCAGGAGCTAA	this study
KAN1	27	51	GGGAGATCTGCTTTTTAGACATCTAAA	this study

[a] Tm (°C) is calculated as Tm(°C)= 2(A+T) + 4(G+C) from Itakura *et al*, 1984

Pre-Amplification Denaturation	92°C	1 minute	
Denaturation	92°C	1 minute	
Annealing	47°C	1 minute	30 cycles
Elongation	72°C	2 minutes	

2.7.1 Colony PCR

Colony PCR was performed on *H. pylori* and *E. coli* transformants to screen for the plasmid pAB03 (Section 3.3.2). Colonies were picked using sterile pipette tips and suspended in 2µl of 1 x PCR buffer. 1µl of this suspension was added to each of the PCR reactions. Denaturation lysed the cells, releasing the DNA, and allowing amplification.

2.8 Cloning techniques

Plasmids were constructed by isolating DNA from digests or PCR reactions, ligating with plasmid vector, and transforming the ligation reaction into *E. coli* by means of heat shock or electroporation. Plasmids were also transformed into *H. pylori* by means of natural competence.

2.8.1 Isolation of DNA

DNA to be cloned was produced either by a restriction digest or through PCR amplification. DNA obtained from restriction digests (Section 2.6.1) was size fractionated by electrophoresis in a 1% agarose gel. The correct sized band was excised and gel purified using the Gel Purification Kit (Qiagen). DNA amplified by PCR was checked by size fractionating 1µl of PCR reaction by electrophoresis. If non-specific bands were present, the remainder of the reaction was size fractionated and the correct sized band was excised from the gel and gel purified. If no non-specific bands were present, however, the PCR reaction was purified using the High Pure PCR Product Purification kit (Boehringer Mannheim). 1µl of the purified DNA was then size fractionated by electrophoresis to estimate the concentration.

For DNA that was too low in concentration to gel purify, ligations were carried out in the gel slice. The DNA was sized fractionated by electrophoresis on a 1% SeaPlaque low melting point agarose gel. The relevant band could then be excised.

2.8.2 Ligation reactions

Vector:insert ligations were carried out with both blunt and cohesive ended fragments of DNA. Both ligation types were made up with approximately a 1:3, vector to insert molar ratio. T4 DNA ligase was added to the ligation reaction to give a final concentration of 1 unit/10µl, while ligase buffer was added to give a final concentration of 1 x ligation buffer. The reactions were made to a final volume of 10µl with sterile distilled water and incubated

for 1-2 days. The incubation temperature depended upon the type of ligation reaction. Blunt ended ligation reactions were incubated at 12°C or room temperature, while cohesive end ligation reactions were incubated at 4°C or 12°C.

Ligations were also carried out in gel slices. This eliminated the need to gel purify DNA which often resulted in low yields. The gel slice was melted and the desired quantity was removed to a warm PCR tube. Ligation buffer and insert DNA were added at this time. The solution was then reheated to ensure that the gel was liquid, and then the T4 DNA ligase was added. Incubation was carried out at room temperature.

Three-way ligations were also carried out that involved the ligation of a plasmid vector and two antibiotic markers. The molar ratio of vector to insert to insert was 1:2:2. Incubation was as described for blunt ended and gel slice ligation reactions.

2.8.3 Transformation into *E. coli*

Aliquots of calcium competent *E. coli* are kept at –80°C for laboratory use. Cells were thawed on ice and 100µl mixed with 5-15µl of ligation reaction. The cells were then kept on ice for 30-60 minutes with occasional mixing. The cells were then heat shocked at 42°C for 30 seconds and then chilled on ice for 5 minutes. They were then transferred into 2mls of Luria broth and shaken at 37°C for 30 minutes. After this time, the cells are plated on selective plates and incubated over night at 37°C. If blue/white colour selection was available (ie: ligating into pUC18/19), the cells were plated on X-gal plates (Luria agar, 5mM IPTG, 80mg/ml X-gal, and 100mg/ml ampicillin).

2.8.4 Transformation by electroporation

Electrocompetent *E. coli* ER2206 cells were prepared by inoculating 50mls of Luria Broth suplemented with tetracycline with 0.5ml of an overnight *E. coli* culture. The cells were grown until the OD_{600} = 0.5-0.8. The flask was then chilled in an ice-water bath for at least 30 minutes and the cell suspension then centrifuged at 4°C in the Sorval refrigerated centrifuge at 5000rpm for 10 minutes. The SS34 rotor was chilled prior to centrifugation. The supernatant was decanted and the pellet resuspended in 50ml of ice cold sterile water. The suspension was then centrifuged again at 5000rpm for 10 mins and the supernatant was decanted. The cell pellet was then resuspended in 80µl of ice cold sterile water and kept on ice until use.

Whilst the electrocompetent cells were being prepared, the ligation reacton was microdialysed on a section of glass filter against 10% glycerol to remove salts from the mixture which may cause electrical arcing during electroporation. Dialysis took 1-2 hours. 25µl of electrocompetent cells were diluted two-fold and the cell suspension was mixed with 3-4µl of the ligation reaction and electroporated at maximum voltage (2.5V). The time constants for the electroporation average 4.6 with a range of 4.5-4.7. The electroporated cells were then suspended in 2mls of Luria broth and shaken at 37°C for 30 minutes. The cells are then plated on selective plates and incubated over night at 37°C.

2.8.5 Transformation of plasmids into *H. pylori*

Transformation of plasmid DNA into *H. pylori* relies upon natural competence. A two day old plate of *H. pylori* was harvested by removing the bacteria with a damp swab and

resuspending in 1ml of CO_2 equilibrated Tryptone Soya Broth. The optical density of the suspension was then measured and the suspension diluted to give an OD_{600} of 0.4-0.6. 200µl of the cell suspension was then transferred to a sterile microfuge tube with a screw top lid. 2µg of plasmid DNA was then added to the cells and the tube was placed in the CO_2 incubator with the lid loosened. The tube was gently shaken every half hour for four hours, at which time the cells were plated on a non-selective CBA plate. After 24hrs of growth, a thin layer of biomass was harvested and resuspended in 1ml of CO_2 equilibrated TSB. This was then centrifuged and the supernatant removed. The pellet was resuspended in the remainder of the supernatant. 1µl of the suspension was diluted 10^5-fold and plated on a non-selective CBA plate to indicate the number of receptive cells. The remainder of the suspension was plated on a selective plate and incubated at 37°C for 4-5 days.

2.9 Southern blotting and hybridisation

2.9.1 Preparation of probes

Probes for Southern blotting were produced by PCR amplification, incorporating dig-dUTP into the DNA sequence. The probe was then purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim). A titration was then performed using decreasing concentrations of the probe and spotting 5µl onto a piece of Hybond-N^+ filter. The filter was then subjected to the detection washes as described in the Dig System Manual (Boehringer Mannheim) and exposed to X-ray film for 7 minutes. The optimal concentration of the probe was then determined so that the correct amount of probe could be added to the membrane

during hybridisation (Section 2.9.4) which would produce clear bands with a minimum background.

2.9.2 Preparation of DNA for Southern blotting

DNA to be blotted was prepared by digesting genomic DNA with a frequently cutting restriction enzyme such as *Hind*III or *Eco*RI, which would produce a smear when size fractionated by electrophoresis. The 1% agarose gel was then photographed under UV light and the standard molecular weight markers were traced on to a piece of overhead transparency.

2.9.3 Southern blotting

The gel was washed twice for 5 minutes in 0.32% hydrochloric acid, twice for 20 minutes in 1.5M NaCl/0.5M NaOH and then twice for 20 minutes in 1.5M NaCL/1M Tris. The gel was then dipped in 2 x SSC. The blot was assembled as follows. A tray from an electrophoresis chamber was turned upside down in a shallow dish and covered with a wick of Whatman filter paper prewetted in 2 x SSC. The dish was filled with 20 x SSC. The gel was then carefully placed on the wick and on this was placed a piece of Hybond-N^+ membrane prewetted in 2 x SSC, cut to the same size as the gel. Any bubbles were carefully removed to ensure blotting of all DNA. Glad wrap was placed around the gel to prevent the movement of liquid through any material other than the gel and membrane. Four pieces of Whatman filter paper prewetted in 2 x SSC were then laid on top of the membrane and any bubbles were carefully removed. A stack of paper towels were then placed on top of the filter paper and then a glass plate was

placed on top of the towels. Finally, a weight was placed on top of the plate and carefully centred to ensure even weight distribution and, therefore, even blotting. The blot was left overnight to allow the transfer of the DNA.

2.9.4 Hybridisation and detection of Dig-dUTP labeled probes

After the blot had been left to stand overnight, it was dismantled and the membrane was marked with the molecular weight markers using the overhead transparency. The membrane was then rinsed in 2 x SSC and wrapped in Glad wrap. The membrane was cross-linked by placing the membrane DNA side down on a transilluminator and exposing to UV light for three minutes. After cross-linking, the membrane was unwrapped and left to dry on a piece of Whatman filter paper.

The hybridisation and detection were carried out based on the method described in the Dig System User's Guide for Filter Hybridisation (Boehringer Mannheim). The membrane was then exposed to X-ray film for 10 minutes and the film developed using the standard procedure. This consisted of immersing the film in developer for five minutes, rinsing briefly in water, and then immersing in fixing agent for a further five minutes. The film was then rinsed in water and air dried.

2.10 DNA Sequencing

Sequencing was achieved through manual (Section 2.9.1) and automatic (Section 2.9.2) sequencing reactions using custom primers (Table 2.2). Limited sequence of *csg* and *glnP1* was available at the onset of the study which was also produced through manual and automatic sequencing.

2.10.1 Designing of sequencing primers

Sequencing primers were designed to contain an approximately equal proportion of A+T to G+C. This kept the T_m of the primer at approximately 55°C, although this varied slightly depending on the length of the primer. Primer length was generally kept to 18-21 nucleotides. All primers are shown in Table 2.2.

2.10.2 Manual sequencing

2.10.2.1 Preparing the acrylamide sequencing gel

The acrylamide gel was prepared by mixing 33g urea, 33ml water, 7.7ml 10 x TBE, 0.37ml 10% APS, and 13ml Acrylamide/Bis until the solution was fully dissolved and had reached room temperature. During this time, the two glass plates were cleaned and rubbed with 2% Silane on the sides which would be in contact with the gel. The gel mould was then assembled

and held together with a casting boot (Biorad). 37µl Temed was then added to the gel solution and the mixture was carefully injected into the mould using a 60ml syringe. Any bubbles were removed with a flat hook. The combs were then inserted and the gel was left to polymerise. Once the gel had polymerised, the casting boot was removed and the gel placed in the sequencer. 1 x TBE was added to both the upper and lower reservoirs of the sequencer and the combs were removed. Any bubbles in the wells were removed by gently pipetting 1 x TBE into the wells.

2.10.2.2 Sequencing reaction

Manual sequencing was performed using the Ampicycle Sequencing Kit (Amersham). This method uses linear amplification of DNA with the incorporation of ddNTPS and 35^S labeled dCTP. Cycle sequencing was carried out as described in the Ampicycle manual. Each tube was sequenced in the presence of one of ddATP, ddTTP, ddCTP, or ddGTP. After the cycle sequencing was complete, 4µl of reaction stop mixture was added to each of the four tubes. The samples were then boiled for three minutes to denature the DNA, and the half of the reaction mixture was loaded onto the sequencing gel. The sample were size fractionated by electrophoresis at 1750-2000V until the more retarded of the two dye bands (Xylene) had reached the bottom of the gel. The remainder of the reaction mixture was then boiled and loaded on to the sequencing gel. Electrophoresis was continued until the less retarded of the two dye bands (Bromophenol Blue) had reached the bottom of the gel.

2.10.2.3 Fixing the gel and detection of radioactive DNA sequence

Once electrophoresis was completed, the gel was removed from the sequencer and the upper glass plate was carefully removed. The gel was then submerged in DNA sequencing fixer for 20 minutes. A piece of Whatman filter paper was then cut out to a size slightly larger than that of the gel. The gel was removed from the fixing solution and the filter paper was carefully placed on top of the gel. The plate was then carefully turned over and removed so that the gel was then on the filter paper. The gel was then transferred to a gel dryer for two hours at 80°C to dry the gel. The dry gel was then exposed to X-ray film for three days and developed using standard developing procedures.

2.10.3 Automated sequencing

Automatic sequencing was carried out by the Waikato Sequencing Facility at Waikato University and the Massey University Sequencing Facility (MuSeq). DNA was provided at a concentration of 200µl and primers were provided at a concentration of 0.8ng/µl. Each reaction yielded 500-700 nucleotides of sequence, which was subsequently analysed using Gene Jockey and Gene Works 2.5 software.

2.11 Isolation of RNA

2.11.1 Precautions taken to avoid RNAse contamination

All glassware was sterilised by autoclaving at 15lb of pressure for 15 minutes followed by baking at 180°C for three hours. All solutions were made from chemical stocks reserved specifically for procedures involving RNA and made to volume using water treated with DEPC. The DEPC-water was incubated for 24 hours and then autoclaved. At all times, powderless gloves were worn. All working surfaces were wiped with β-mercaptoethanol or RNAse AWAY (Molecular Bio-Products) to inactivate residual RNAse.

2.11.2 Isolation of RNA using TRIZOL reagent

10ml samples of a *H. pylori* liquid culture were collected at morning and evening time points. The cells were pelleted by centrifugation at 5,000rpm for 5 minutes. 0.5ml of TRIZOL reagent (Life Technologies) was added to the pellet and the cells were lyzed by repetitive pipetting. The homogenised sample was incubated for five minutes at room temperature, at which time 0.2ml of chloroform was added. The tubes were shaken for 15 seconds and incubated for 2-3 minutes at room temperature. The samples were then centrifuged at 12,000 x g for 15 minutes. The colourless aqueous phase was transferred to a fresh microfuge tube and 0.25ml of isopropanol was added to precipitate the RNA. The samples were incubated for 10 minutes at room temperature, then centrifuged at 12,000 x g for 10 minutes. The supernatant was then

removed and the pellet was washed with 70% ethanol. The samples were mixed by vortexing and centrifuged at 7,500 x g for five minutes. The RNA pellet was air dried for 5 minutes and resuspended in 50µl of DEPC-water. The RNA suspension was heated at 60°C for 10 minutes to allow the precipitated RNA to go into solution. RNA solutions were stored at -80°C.

2.11.3 Treatment with RNAse-free DNAseI

5µl of RNA was treated with RNAse-free DNAseI. Vanadyl ribonucleotide complex (VRC) was added to the RNA to give a 20-fold dilution. 1µl of DNAseI was added to the RNA and the solution was incubated for 15 minutes at 37°C. The DNAseI was then heat killed at 75°C for 10 minutes.

2.12 Northern blotting

2.12.1 Electrophoresis of RNA

RNA was size fractionated by gel electrophoresis in a 1.2% agarose formaldehyde gel. 0.6g agarose, 1ml 50 x MOPS EA, and 46.5ml DEPC-water were mixed and heated until the agaorse had dissolved. The gel mixture was then cooled to 55°C and 2.5ml 37% formaldehyde was added. The gel was poured into a mould cleaned with DEPC-water and RNAse AWAY. The samples were mixed with a equal volume of RNA Final Solution Buffer (FSB), heated to 65°C for 15 minutes and cooled on ice for 10 minutes. 4µl 250mg/ml

ethidium bromide was added to the samples, which were loaded on the gel. A voltage of 90V was applied to the gel in 1 x MOPS EA running buffer.

2.12.2 Preparation of probes

Probes were produced which were labeled for either Dig (Boehringer Mannheim) or ECL (Amersham) detection systems. Dig probes were prepared by PCR amplification of target DNA with the incorporation of dig-dUTP. The probes were tested for concentration as described in Section 2.9.2.

ECL probes were labeled after PCR amplification of the target DNA and directly prior to use. DNA was diluted to 10ng/µl. 10µl of the diluted probe was then denatured by boiling for 5 minutes. An equal volume of DNA labeling reagent was then added to the probe, then 10 µl of gluteraldehyde solution. The probe was mixed thoroughly. The solution was incubated for 10 minutes at 37°C and then held on ice until required (usually 10-15 minutes).

2.12.3 Northern blotting

After electrophoresis, the agarose gel was photographed under UV light. All surfaces were wiped with RNAse AWAY. A section of overhead transparency was used to mark the molecular weight markers that would be detected after blotting. The gel was then washed three times for five minutes in DEPC-water, then for 40 minutes in 10 x SSC. The northern blot was then set up and carried out as described for Southern blotting (Section 2.9.2).

2.12.4 Slot blotting

Slot blotting was used to confirm the presence of target RNA in the samples obtained from liquid cultures. As no electrophoresis is involved, no information was yielded regarding the size or quantity of the target RNA. A vacuum is used to draw the RNA solutions through the membrane, leaving the RNA bound to the membrane. An 8 x 11cm piece of Hybond-N^+ membrane was briefly wetted in DEPC-water and then soaked in 20 x SSC for 1 hour. The slot blot apparatus was cleaned with 0.1M sodium hydroxide, then rinsed with DEPC-water. Three pieces of Whatman 3MM paper were placed on the vacuum unit and wet with 20 x SSC. The membrane was carefully laid on top of the paper so as to exclude any bubbles. The lid was then screwed on. All slots were filled with 10 x SSC and drawn through by vacuum. They were then refilled. 5µl RNA was mixed with 5µl DEPC-water, 20µl 100% formamide, 7µl 37% formaldehyde, and 2µl 20 x SSC. The solution was incubated at 68°C for 15 minutes and then cooled on ice. Two volumes of 20 x SSC were added to the samples which were then applied to the slots. The samples were then drawn through the membrane under vacuum. The slots were then washed twice with 10 x SSC and the suction then left on for 5 minutes to dry the membrane.

2.12.5 Hybridisation and detection of RNA probes

Membranes were crosslinked by baking for 2 hours at 80°C. The blots were then hybridised by the Dig or ECL method.

2.12.5.1 Hybridisation of Dig-dUTP labeled probes

This method is based on that described in the Dig System User's Guide to Filter Hybridisation (Boehringer Mannheim).

2.12.5.2 Hybridisation of ECL labeled probes

This method is based on that described in the ECL manual (Amersham).

2.13 Growth curves

2.13.1 Inoculation of liquid cultures

H. pylori from two day old plates were harvested and used to inoculate 15ml of complex medium (Tryptone Soya Broth). The cultures were placed in an anaerobic jar with a CO_2 producing gas pack and incubated for 24 hours. After this time, a 5% inoculation was made into 15ml of complex or defined medium (non-selective or selective). The defined medium was made up to allow the exclusion of one or two components, which were replaced with SDW to maintain the concentration of the remaining components. All media were CO_2 equilibrated prior to inoculation.

2.13.2 Sampling

0.5ml or 1ml samples were taken from *H. pylori* liquid cultures at both morning and evening time points. This meant a 8-9 hour period between samples during the day and 15-16 hour period overnight. Samples were used to estimate total cell counts, to plate for viable cell counts, to measure the pH of the culture, and to measure the optical density of the culture.

2.13.3 Total cell counts

Total cell counts were performed using a cytometer. The cytometer was divided into 25 large squares, each divided into 16 smaller squares. A sample of a culture was diluted such that up to 40 cell were visible in each square and each of the 16 squares in one of the large 25 squares was counted. The number of cells was then multiplied by 2.5×10^5 to give the number of cells per ml.

As the cell shape was clearly visible under magnification, the number of coccoid and helical cell types were counted. This data provided information concerning the conversion of rods to cocci in cultures entering death (or starvation) phase.

2.13.4 Viable cell counts

The *H. pylori* sample was diluted to give 30-300 colonies per CBA plate. This dilution factor ranged from 10^1 to 10^9 depending on the change in the total cell count between two samples.

The diluted culture was plated on selective or non-selective CBA plates and incubated in a CO_2 incubator for 3 days before colonies could be distinguished and counted.

2.13.5 Optical density readings

1ml of each sample was measured at OD_{600} for density in a bench spectrophotometer.

2.13.6 pH measurements

5-10µl of each sample was pipetted onto a strip of Whatman narrow range (pH 6-8) litmus paper. Colour developed within 30 seconds and the pH was read by comparison with the supplied standards.

3.0 Results

3.1 Sequencing of *csg*

3.1.1 Sequence available prior to the onset of the thesis project

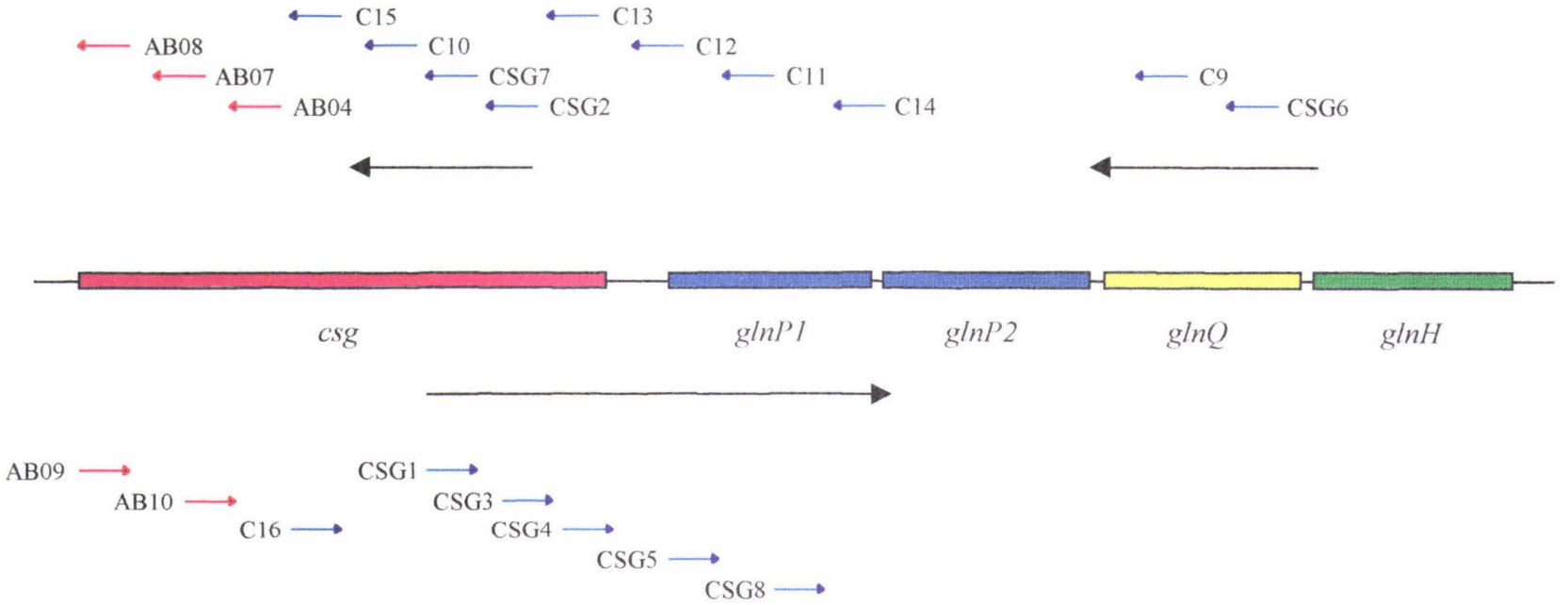
Approximately 1850 nucleotides of sequence was available prior to the onset of this thesis project. The sequence was generated by automated sequencing using five primers, Forward Primer, CSG1, CSG3, CSG4, and CSG5 (Table 2.2), and using pHP040 (Appendix A.1) as the DNA template. A manual sequencing reaction was also performed with another primer, C8 (Table 2.2), to extend the sequence.

The sequence contained partial coding sequence for *csg* (757 nucleotides) and the complete coding sequence of *glnPI* (Fig. 3.1). The two genes are divergently transcribed and, as such, sequence was generated in the 3'-5' direction for *csg*, and in the 5'-3' direction for *glnPI*. The intergenic region is also contained in this block of sequence and was able to provide some information concerning the promoters of both genes. It was noted that both genes had a putative σ^{70} promoter.

Primers CSG2 and CSG7 (Table 2.2) provided limited sequence on the second DNA strand due to proximity of the binding sites to the vector sequence. Reverse primer, CSG6, and C9

Figure 3.1 Schematic diagram showing available sequence and position of primers

The available sequence at the onset of this thesis project is represented by the black lines, with arrows depicting the 5' to 3' direction. Existing primers are represented by the blue lines, while primers from this study are shown in red. Arrows determine the direction of extension.


C15
C13
AB08
C10
C12
AB07
CSG7
C11
C9
AB04
CSG2
C14
CSG6
csg
glnP1
glnP2
glnQ
glnH
AB09
CSG1
AB10
CSG3
C16
CSG4
CSG5
CSG8

(Table 2.2) provided the complete coding sequence of the *glnQ* gene at the reverse primer end of the insert.

At this stage, the DNA template was changed to a second plasmid, pHP042 (Appendix A.2). pHP040 was one of eight clones constructed by Dr Paul O'Toole (unpublished data) which were thought to contain genes important in cellular metabolism. To investigate whether or not additional csg sequence was present on the remaining seven clones, sequencing was performed using primer CSG7, which extended the *csg* sequence from 5' to 3'. One of the remaining seven clones yielded *csg* sequence and was subsequently named pHP042.

Primer C10 extended sequence from the CSG7 sequencing reaction using pHP042. Primers C11-14 (Table 2.2) were designed from the block of sequence to complete the double stranded sequence but were not used in this capacity until this thesis project had begun. Likewise, primers C15 and C16 were designed to extend the C10 sequence and complete the double stranded sequence, respectively, but were not used until the project had begun. A schematic diagram of priming sites is shown in Fig. 3.1.

3.1.2 Sequencing of *csg* and *glnP1*

Primers C11, C12, C13, and C14 were used to complete the double stranded sequence of *csg* and *glnP1* (Appendix B.1) available on pHP042. This was achieved through manual sequencing and later, along with CSG2 and CSG7, through automated sequencing. The two contiguous sequences of 2022 nucleotides were identical but for one nucleotide, found to be

an error in the automated sequence reading. This sequence was submitted to GenBank on 10 December, 1996 and was assigned the accession number U81796.

By sequencing the insert of pHP042 with Forward and Reverse primers, it was discovered that pHP042 and pHP040 contained almost identical genomic fragments. pHP042 differed from pHP040 in that the partial sequence of *csg* was approximately 400 nucleotides longer, and that the *glnQ* gene was truncated and fused to a 156bp fragment of the *fliI* gene.

Sequence using primer C10 was found to be comprised of *csg* and vector sequence. As all *csg* sequence had been obtained from the available plasmids, it was necessary to construct a new sequencing plasmid by cloning the *csg* gene from the *H. pylori* genome.

3.1.2.1 Isolation of the 3' end of csg

To be able to clone the *csg* gene from *H. pylori*, it was first necessary to locate the gene genomic digests. This was achieved by Southern blotting (Section 2.9). A probe of 300 nucleotides in length was made utilising two existing sequencing primers to amplify a fragment of *csg* by PCR (Section 2.6). Primers CSG1 and C13 (Table 2.2) were used in a PCR reaction which also contained Dig-dUTP. The Dig-dUTP was incorporated into the amplified fragment during PCR, thus producing and labelling the probe simultaneously. Despite the use of sequencing primers for the PCR amplification, no non-specific fragments were produced and the remainder of the PCR reaction was purified by an ethanol precipitation (Section 2.5.6).

H. pylori genomic DNA was digested with one of three restriction endonucleases; *Hind*III, *Bam*HI, and *Eco*RI. The three digest reactions were then size fractionated by gel electrophoresis and transferred to a nylon membrane by Southern blotting (Section 2.9.2). The membrane was then probed using the Dig labelled probe previously described and the probe detected using the Dig system detection reagents. The resulting autorad revealed a single band in the genomic digests (result not shown). This fragment corresponded to the *Hind*III fragment containing the *csg* promoter and *glnP1* sequence. Investigation into the probe sequence revealed a *Hind*III recognition site which would allow the probe to bind to two adjacent genomic *Hind*III fragments. The band detected was of the size expected for the *csg* promoter region and *glnP1* sequence. The desired fragment containing the 3' end of *csg*, however, was not detected. Due to the incorporation of the Dig-dUTP during PCR amplification, it was not possible to cleave the probe with *Hind*III. This would have allowed the isolation of the 100bp fragment which would bind to the desired *Hind*III fragment. This line of investigation was terminated as it was at this time that Glaxo Wellcome supplied sequence from a clone containing *csg*.

3.1.2.2 Construction of a new sequencing plasmid.

It was known that the *H. pylori* sequence had already been fully sequenced but the sequence not released to the scientific community. Glaxo Wellcome UK Ltd was approached to request the sequence of *csg* but the clone containing *csg* had only been sequenced using the forward and reverse primers. The Glaxo *csg* sequence did not extend the sequence already obtained through our own sequencing attempts. Unrelated sequence downstream of *csg* was also

provided, however, from which a primer, AB03 (Table 2.2), was designed that, with the existing sequencing primer CSG2, would amplify 2.6kb of DNA containing the 3' end of *csg*.

The original PCR reaction was performed using *Taq* DNA polymerase and the fragment purified by gel purification. Attempts were then made to clone the fragment into the commercially available vector, pGEM-T (Promega), which possesses 5'-T overhangs cohesive for the 3'-A overhangs generated by *Taq* DNA polymerase. This cloning strategy, however, proved unsuccessful. Blue/white colour selection was available to screen the transformant colonies but all white colonies screen by the quick alkaline plasmid preparation (Section 2.5.2) were shown to possess a small deletion rather than an insertion of 2.6kb. After several further unsuccessful attempts, the PCR amplification was repeated with *Pwo* DNA polymerase which resulted in a blunt ended product. The product was purified by gel purification after gel electrophoresis, and cloned into pUC19 cleaved with *Sma*I. Blue/white colour selection was again available to screen the transformant colonies. A plasmid preparation of several of the white colonies showed the transformants to contain an insert of the expected size.

To confirm the successful cloning of the PCR product, PCR amplification was used. Reactions were made up with either forward or reverse primer and a primer internal to the insert (C15). A positive result was obtained in the reaction using forward primer. This one reaction confirmed the presence of the insert, and determined the orientation of the insert within the vector. The plasmid was named pAB01 (Appendix A.4).

It was at this stage of sequencing that the complete *H. pylori* 26695 genome was released on 25 July, 1997, on the internet by The Institute for Genomic Research (TIGR). Sequencing of

csg continued, however, to determine the *csg* sequence homology between the two strains of *H. pylori*.

pAB01 was used as DNA template for automated sequencing reactions with AB04, AB07, AB08, AB09, and AB10 (Table 2.2) to complete the sequence of *csg* in both the complementary and coding strands of the DNA. The reactions were carried out by the Massey University DNA Sequencing Facility (MuSeq). The sequence is shown in Appendix B.2.

3.1.3 Analysis of the *csg* sequence

The sequence of *csg* was found to be 2061 nucleotides in length, which corresponded to a protein of 687 amino acid residues. The promoter region of *csg* was found to have putative σ^{70} -10 and -35 consensus sequence (Fig. 3.2). The *E. coli cstA* promoter is also a σ^{70} responsive promoter as determine by promoter mapping (Schultz and Matin, 1991). However, *cstA* does not have a -35 consensus sequence. A putative CRP binding site also exists in the promoter region of *csg* (Fig. 3.2). As has been demonstrated in *E. coli*, a large distance exists between the putative transcription start point, and the CRP binding site. Schultz and Matin (1991) have suggested the possibility of another factor which may bind in the space between the transcription start point and the CRP binding site but such a factor has yet to be discovered.

The amino acid sequence was found to contain multiple hydrophobic domains as shown in the hydropathicity plot (Fig. 3.3) generated by the algorithms of Kyte and Doolittle. The

Figure 3.2 Sequence of *csg* and *glnP1* showing the putative promoters of *csg*

Sequence available at the onset of the thesis project is shown. The putative csg σ^{70} -10 and -35 sequences are underlined in red. The signal sequence is shown in green. A putative CRP binding site is shown boxed in red and can be seen to be located in the 5' end of the *glnP1* sequence. This forms the basis for the work discussed in Section 3.2.

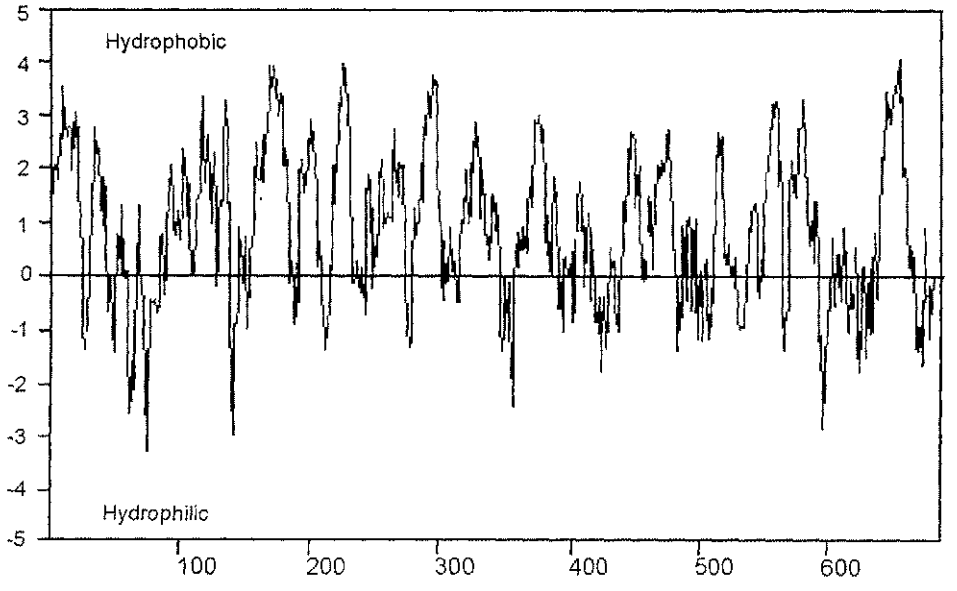
```
AAAACCCCAC CAAAAGAGAG AGCAAAATTC CAAAGAAAGA AATATAAAGC     50
 ehPylGlaV ueLueLreSu eLueLellyl GehPehPreS ellryTueLr
GTGAGTTCTA ACCCCTTAAA AAACGCAGGG ATGGAGTGAA ACATAAAATC    100
hTueLulGue LylGsyLehP ehPalAorPe IlreSsiHeh PteMehPpsA
CCAATCTAAT GCCATAACGC TATACCTTAA ATTAATGACG CTTTAAAGCA    150
prTpsAueLa lAteM
CCATGTTTAA AAACAGCTCT TATTCACCCT AATTGTTAGA AGTTTGGAAA    200

ACCCAAGCCC TTATTATGGG AAGTTTATGA GCTATTTTGC CACTATTCAA    250

AGAAAATTTG AATTATAATA AGAAACTGGC TGAAATTAAC AACAATGATT    300
                                                  MetIle
AAACAATCAT TAAATGGAGA GGACATGCAA AAAAGTTTAG TTTCTTTGGC    350
LysGlnSerL euAsnGlyGl uAspMetGln LysSerLeuV alSerLeuAl
TTGGGTTTTT GTCGCTATTT TAGGGGCGAT CTGTTTAGGG GTGTTAGCCT    400
aTrpValPhe ValAlalleL euGlyAlall eCysLeuGly ValLeuAla.
```

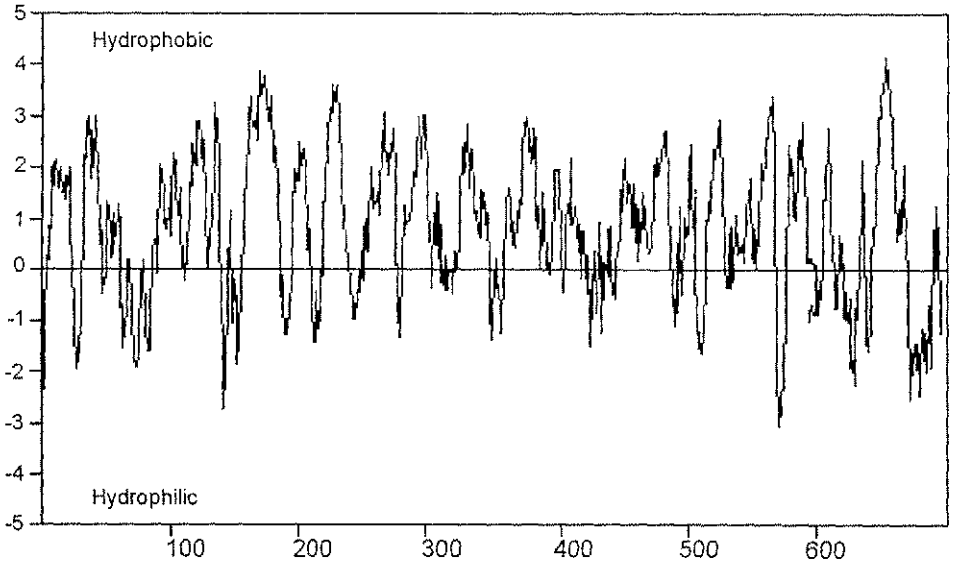
Figure 3.3 Hydropathicity plot of Csg

The hydropathicity plot shows all hydrophobic domains as peaks above the centre line, while peaks below the centre line represent hydrophilic domains. Groups of six amino acids are used to determine each point.

Figure 3.4 Hydropathicity plot of CstA

The hydropathicity plot of CstA is very similar to the of Csg. This implies that the two proteins have similar localisations


Hydrophobic
Hydrophilic
5
4
3
2
1
0
-1
-2
-3
-4
-5
100
200
300
400
500
600


Hydrophobic
Hydrophilic
5
4
3
2
1
0
-1
-2
-3
-4
-5
100
200
300
400
500
600

hydropathicity plot was also shown to be very similar to that of *E. coli* CstA (Fig. 3.4). This demonstrates the possible similarity in localisation between the two homologous proteins as the presence of the hydrophobic domains suggests that the proteins are located in the membrane.

The G+C content of *csg* is higher than the average for the *H. pylori* 17874 genome. The genome has an average G+C content of 35.6%, as determined by thermal denaturation (Béji *et al*, 1988). This method of estimating the G+C content gave a range of 34.1-37.5% for 20 *H. pylori* strains - the type strain and 19 clinical isolates. This method is not as accurate as sequencing the genome to obtain the data, but such a method has not been applied to *H. pylori* 17874. The G+C content of *H. pylori* 26695 is 39%, which may indicate that the values calculated by Béji *et al* (1988) are lower than the true values. *H. pylori* 17874 *csg* has a G+C content of 44.5%, significantly higher than that of the genome. This may imply that the *csg* gene was acquired by *H. pylori* from another source.

Comparison of the *H. pylori* 17874 *csg* sequence and the released *H. pylori* 26695 *csg* sequence revealed several minor differences. *H. pylori* 17874 *csg* exhibited 96.4% identity at the nucleotide level to *H. pylori* 26695 *csg*, but as most of the differences were nucleotide changes in the third position of the codon, the amino acid residue remained unchanged. Several base changes which were not in the third position of the codon did alter the amino acid sequence, resulting in 98.1% identity at the peptide level between the two strains. The differing amino acid still had the same chemical properties, ie; remained neutral, basic, or acidic, however, two of the 687 amino acids (position 424 and 623) differed in their chemical properties.

3.2 The study of linkage between *csg* and *glnP1*

Analysis of the *csg* sequence revealed a putative cAMP receptor protein (CRP) binding site within the 5' sequence of *glnP1*. The implication is that *csg* and *glnP1* are linked as *glnP1* sequence contains a possible regulator of *csg*. This linkage may be significant as Jaing *et al* (1996) reported that high diversity exists in the gene order between strains of *H. pylori*.

As *csg* homologues have been described in very few bacteria, it was only possible to examine the linkage between *cstA* and *glnP* in *E. coli*. Using the *E. coli* Genome Collection (ECGC) database, it was possible to observe that there is to be no linkage between the two genes. This is indicated by the gene maps, demonstrating that the two genes map to different regions of the genome. *cstA* is mapped to 13.56 minutes, while *glnP* is mapped to 18.23 minutes.

It was also found that the glutamine permease operon in *E. coli* is arranged differently to that of *H. pylori*. The *E. coli* operon is arranged as *glnH*, *glnP* and *glnQ* (Fig. 3.5). *glnPQ* are transcribed via the same σ^{70} promoter whereas *glnH* is under the control of a σ^{54} promoter. In *H. pylori*, the glutamine permease operon is arranged as *glnP1*, *glnP2*, *glnQ*, and *glnH* (Fig. 3.6).

3.2.1 PCR amplification of the *csg* - *glnP1* 5' sequence and intergenic region

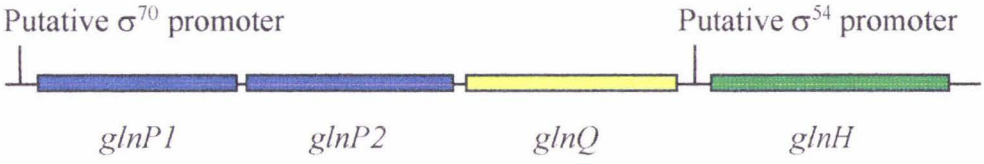
Comparison of the sequence of the *H. pylori* 17874 *csg-glnP1* intergenic region with that of *H. pylori* 26695 (released by TIGR) shows that the distance between the two genes differs by just three nucleotides between the two strains. To test the hypothesis that *csg* and *glnP1* are

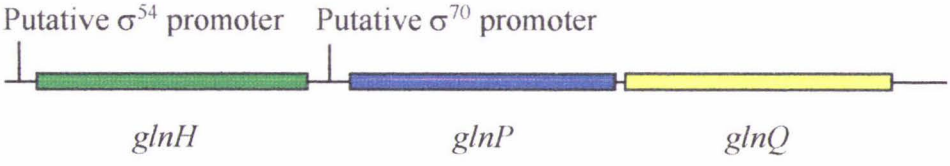
Figure 3.5 The organisation of the *H. pylori* glutamine permease operon

The glutamine permease operon of *H. pylori* has two *glnP* homologues and the *glnQ* homologue, each separated by just one nucleotide, that are under the control of a putative σ^{70} promoter. The *glnH* homologue is downstream of *glnPQ* and is regulated by a putative σ^{54} promoter.

Figure 3.6 The organisation of the *E. coli* glutamine permease operon

The *E. coli* glutamine permease operon has just a single *glnP* gene and is directly upstream of the *glnQ* gene, as seen in the *H. pylori* operon. *glnP* and *glnQ* are under the control of a σ^{70} promoter. The *E. coli* operon differs markedly to that of *H. pylori*, however, in that the *glnH* gene is upstream of the *glnPQ* grouping. The *glnH* promoter is a σ^{54} regulated gene.


Putative σ70 promoter
Putative σ54 promoter
glnP1
glnP2
glnQ
glnH


Putative σ54 promoter
Putative σ70 promoter
glnH
glnP
glnQ

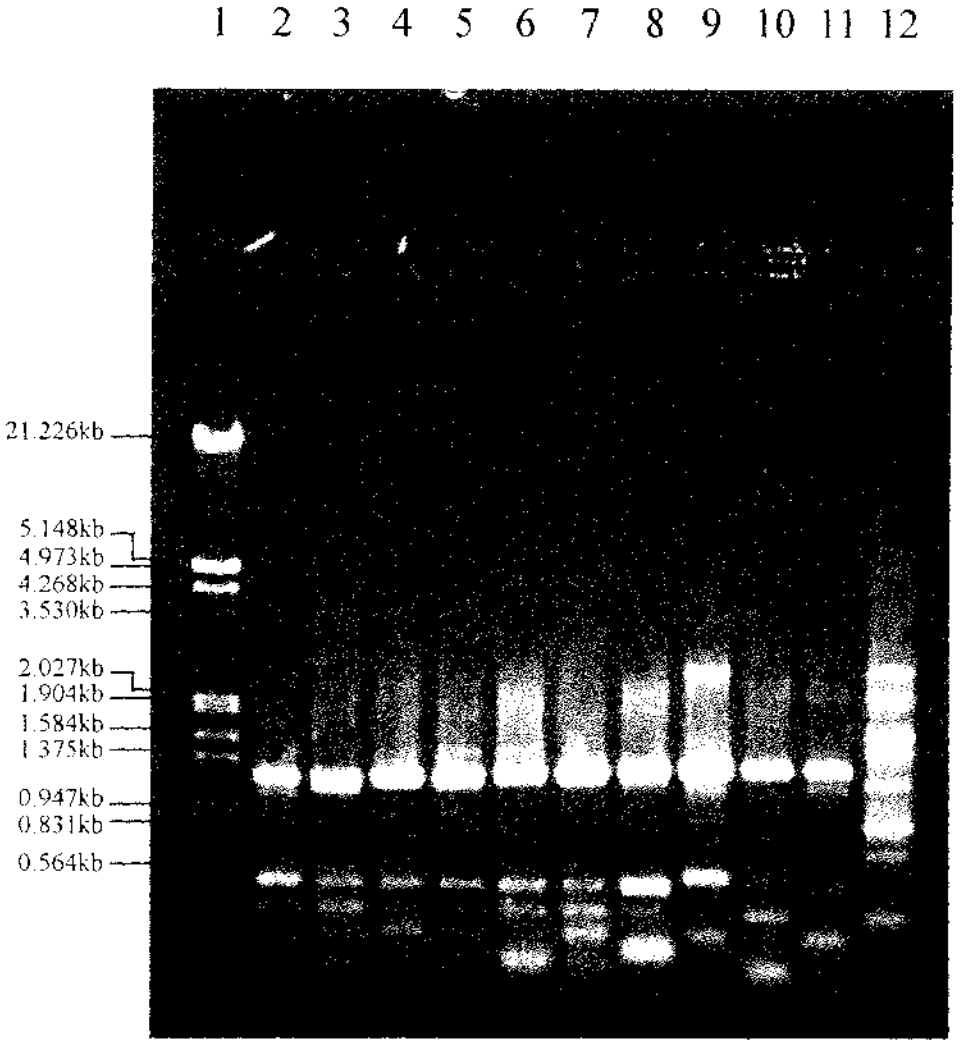
linked in *H. pylori*, PCR (Section 2.7) was used to amplify a region containing the 5' sequence of *csg* and *glnP1* and the intergenic region from ten different *H. pylori* strains. Primers CSG3 and C14 (Table 2.2) were used for the amplification. CSG3 binds to a site internal to *csg* while C14 binds to site internal to *glnP1*. The resulting PCR product would represent the distance from gene to gene rather than an amplified fragment of the intergenic region. The template for the reactions was the genomic DNA of strains *H. mustelae* 4298, *H. pylori* 17874, and 9 clinical *H. pylori* isolates (Table 2.1). The PCR reactions were set up as described in section 2.7 with the exception that the magnesium chloride concentration was increased to 3mM. The results of the PCR amplification are shown in Fig. 3.7. All ten *H. pylori* strains produced a PCR product of approximately 1220bp in length, indicating that *csg* and the glutamine permease operon in these *H. pylori* strains are linked. This linkage does not seem to be present in other *Helicobacter* species.

3.3 Mutagenesis of *csg* and phenotypic analysis of the mutant strain

The functional role of *csg* homologues is still vague in its definition. Schultz and Matin (1991) offered the conclusion that *cstA* has a role in increasing the range of carbon sources available to the cell during glucose starvation. More specifically, it may allow the utilisation of peptides as a carbon source. To study the *csg* of *H. pylori* 17874, is was necessary to determine the role of the gene. To achieve this, reverse genetics was used. A deletion-insertion strategy was employed to produce a strain of *H. pylori* with a non-functional *csg*. The differences in phenotype of this strain with the wild type strain would allow the elucidation of the putative role of the gene.

Figure 3.7 PCR amplification of the *csg-glnP1* 5' sequence and intergenic region

The PCR amplification of the intergenic region of ten *H. pylori* strains and *H. mustelae* 4298 was performed using primers CSG3 and C14. Lane 1: λ*Eco*RI/*Hind*III ladder, Lane 2: *H. pylori* MU18, Lane 3: *H. pylori* MU91, Lane 4: *H. pylori* MU57, Lane 5: *H. pylori* MU101, Lane 6: *H. pylori* 55, Lane 7: *H. pylori* MU68, Lane 8: *H. pylori* MU61, Lane 9: *H. pylori* MU62, Lane 10: *H. pylori* MU82, Lane 11: *H. pylori* 17874, Lane 12: *H. mustelae* 4298.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3.3.1 Mutagenesis strategy

The compete mutagenesis strategy is shown in Fig. 3.8.

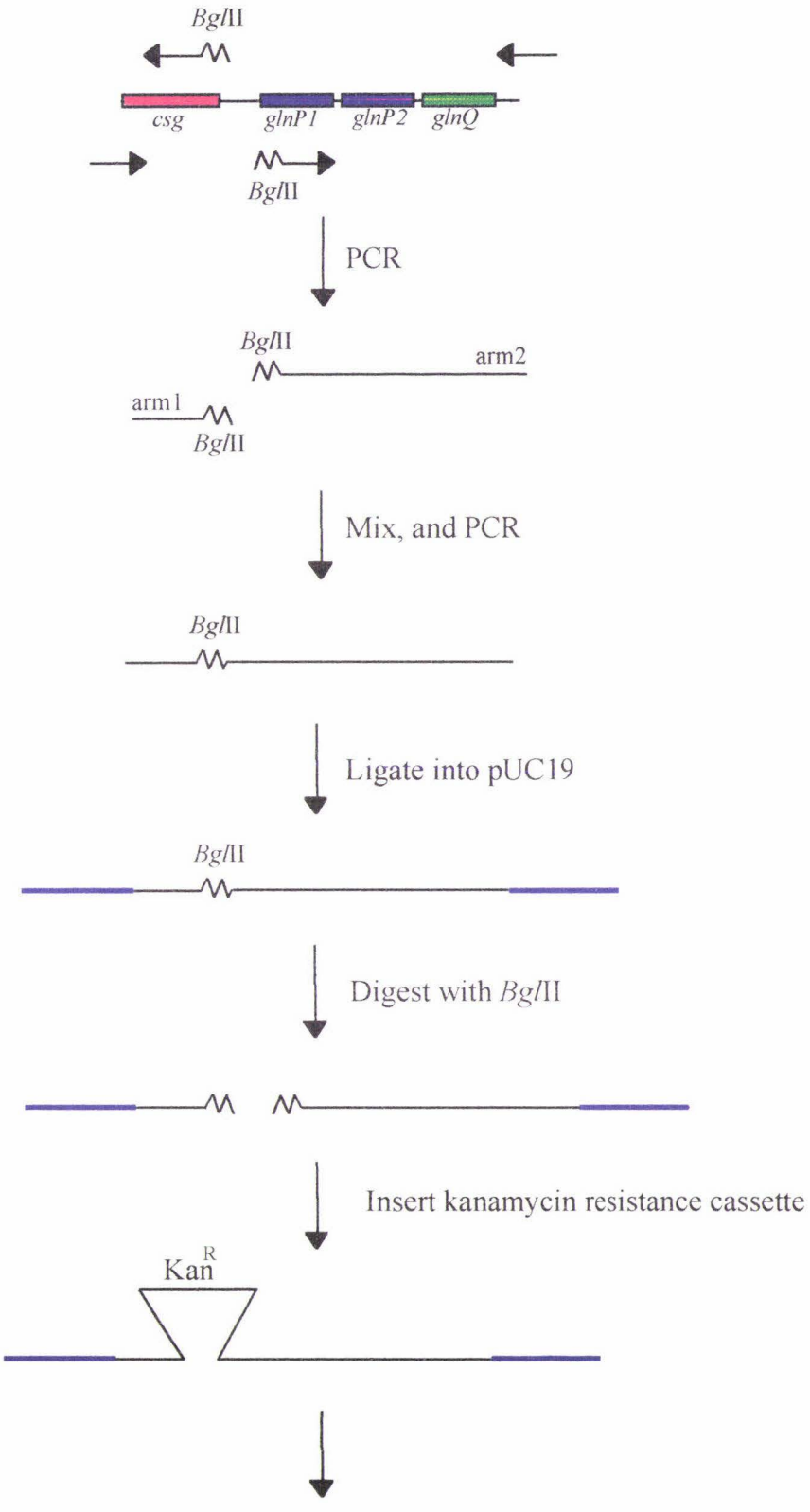
3.3.1.1 Deletion of the csg *promoter region*

To knock out the *csg* gene, it was decided to remove the promoter region and the first 50-100bp of the sequence to ensure that the gene could not be transcribed. As *glnP1* shared the same promoter region, this gene was also knocked out. However, an existing *H. pylori* strain with a non-functional *glnP1* gene (*H. pylori* ΔglnP1) appeared to exhibit no effects from this mutation (P.W. O'Toole, personal communication), and so the production of a double knock out was deemed to be acceptable. Primers AB01 and AB02 (Table 2.2) included *Bgl*II recognition sequences at the 5' ends. AB01 and forward primer (Table 2.2) were used in a PCR reaction (Section 2.7) to amplify a 0.78kb fragment (referred to as arm1) of pHP040 DNA template, while AB02 and reverse primer (Table 2.2) were used in a PCR reaction to amplify a 2.5kb fragment (referred to as arm2). The two fragments were then gel purified. 200ng of arm1 and arm2 were then used as DNA template for a subsequent PCR reaction with forward and reverse primers. PCR amplification resulted in a small yield of the composite fragment which, after gel purification, had a concentration of approximately 20ng/μl.

Ligations were performed using pGEM-T (Promega), which is designed specifically for the cloning of PCR products. Several attempts were made to clone the composite PCR product into pGEM-T. Numerous white colonies were screened by isolating the plasmid DNA using

Figure 3.8 Strategy for the mutagenesis of *csg*

The schematic diagram shows the deletion:insertion strategy for the mutagenesis of *csg*. Two 'arms' were PCR amplified from the plasmid pHP040. Primers AB01 and AB02 have 5' tails that contain *Bgl*II recognition sequences. The PCR of the two 'arms' also excluded a 300bp fragment of DNA containing the promoter regions of both *csg* and *glnP1*. The two 'arms' were then used as template DNA for a subsequent PCR reaction which resulted in a composite fragment with a new *Bgl*II site. This fragment was cloned into pUC19 and a kanamycin resistance cassette then cloned into the *Bgl*II site. The kanamycin cassette was a marker for transformation into *H. pylori*.



Transform into *H. pylori*

the quick alkaline lysis preparation (Section 2.5.2). All colonies screened, however, were found to have a small deletion rather than an insert of 3.2kb.

It was decided to try a blunt-end ligation which required the PCR amplification to be carried out again, using *Pwo* DNA polymerase instead of *Taq* DNA polymerase. It was found that the yield of the composite PCR product was greatly increased by the use of this polymerase enzyme and the resulting concentration after gel purification was approximately 150ng/μl. The blunt-end PCR product was cloned into pUC19 cleaved with *Sma*I. Transformants were screened by blue/white colour selection. Once again, numerous white colonies were screened but were found to have a deletion rather than an insert.

It was suspected that the presence of vector DNA flanking the insert in the PCR product was disrupting cloning attempts in some way, possibly by destabilising the insert. To avoid this problem, the composite fragment was amplified using primers CSG1 and CSG6 which are internal to the pHP040 insert. The composite fragment was then cloned into pUC19 cleaved with *Sma*I. Several white colonies were screened using the quick alkaline lysis preparation (Section 2.5.2) and a transformant with the correct sized insert was found. The presence of the deletion of the promoter region was confirmed by a diagnostic restriction digest with *Bgl*II and the plasmid was named pAB02 (Appendix A.5).

3.3.2 Insertion of a Kanamycin Resistance Cassette

The plasmid containing the mutagenised *csg* gene was to be transformed into *H. pylori*. To be able to select for transformants, however, it was necessary to insert a antibiotic marker. The

kanamycin resistance gene, KanΩ, was chosen as it is flanked by omega fragments which terminate transcription. This prevents the transcription of the remainder of *csg* or *glnP1* (depending on the orientation of the cassette) which might have resulted in a truncated but functional protein.

KanΩ was isolated from pUC4-omega-km2 (Table 2.1) by a *bamb*oo digest. This released a 2.2kb fragment which was gel purified. pAB02 was cleaved with *Bgl*II to produce overhangs cohesive for those produced by *Bam*HI cleavage. A ligation reaction was then performed with linear pAB01 and the KanΩ cassette and the reaction transformed into *E. coli* ER2206. The transformation mixture was plated on L agar plates supplemented with kanamycin (25μg/ml). Four colonies were picked from this plate and the presence of the KanΩ cassette was confirmed by colony PCR (Section 2.7.1). The plasmid was named pAB03 (Appendix A.6).

3.3.3 Transformation of pAB03 into *H. pylori* 17874 and confirmation of transformant structure

H. pylori is not able to maintain *E. coli* plasmids. However, if *H. pylori* genome sequence is present on an *E. coli* plasmid, the DNA can be incorporate into the *H. pylori* genome by means of homologous recombination. This requires a double cross-over event which results in the "swapping" of genomic DNA for plasmid insert DNA. The DNA from the genome is recombined into the plasmid vector and subsequently lost. 2μg of pAB03 DNA was transformed into *H. pylori* 17874 by means of natural competence (Section 2.8.5). The transformed cells were plated on selective CBA plates. 146 colonies grew on the kanamycin plates, indicating the presence of the KanΩ cassette in the genomic DNA. The transformation

frequency was 2 x 10^{-7}, ten-fold lower than expected. However, the transformation frequency with regard to the amount of plasmid DNA used was 1.46 x 10^{2} cells/ μg DNA, which is an expected frequency for *H. pylori* transformation (Segal and Tompkins, 1993).

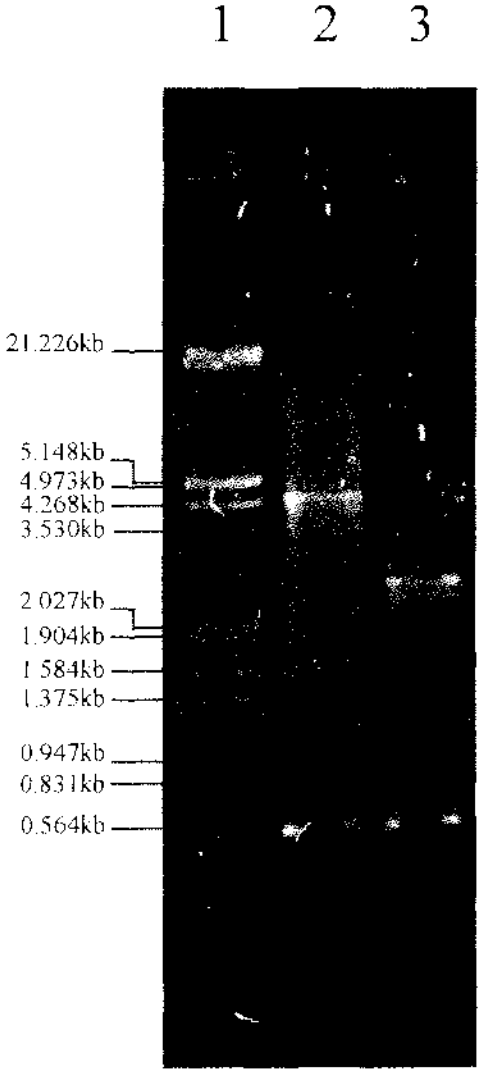
To prove that recombination had occurred at the correct position in the genome, one colony was subcultured twice to ensure purity of the strain. Genomic DNA was then prepared from the strain (Section 2.5.4) for PCR amplification (Section 2.7). Five primer pairs, based on existing sequencing primers, were tested to see if successful amplification would occur. The primer pairs were tested with varying primer and magnesium chloride concentrations (results not shown). CSG3 and C14 (Table 2.2) proved to be the most successful of the primer pairs tested and so PCR conditions were optimised for these primers. The successful conditions were a final magnesium concentration of 2mM and a 5-fold dilution in the DNA template concentration. The resulting PCR products are shown in Fig. 3.9. The lower bands visible in this figure are thought to be non-specific bands as the primers were designed for sequencing and not optimised for a PCR amplification.

3.3.4 Phenotypic analysis of the *H. pylori* Δcsg strain

To be able to analyse the phenotype of the new mutant *H. pylori* strain, media requiring supplementation with blood could not be used. The presence of blood prevents the measurement of optical density of the culture and also contains undefined products that may support growth that would otherwise not occur in the defined medium.

Figure 3.9 PCR confirmation of the *csg* mutation

PCR amplification was performed using primers CSG3 and C14. The photograph shows the *H. pylori* Δcsg PCR product containing an extra 1.9kb of DNA when compared to that of *H. pylori* 17874, corresponding to a deletion of 300bp and the insertion of the 2.2kb kanΩ cassette. Lane 1: λ*Eco*RI/*Hind*III ladder, Lane 2: *H. pylori* Δcsg PCR product. Lane 3: *H. pylori* 17874 PCR product.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3.3.4.1 Phenotypic analysis in complex medium

Tryptone Soya Broth (Section 2.2.6) was used as a complex medium to study the growth characteristics of *H. pylori* 17874 and *H. pylori* Δcsg. The bacteria were able to grow in TSB without the addition of supplements such as fetal calf serum or defibrinated. The aspects of growth observed in this study were the growth rate, the conversion rate from rod to coccoid cell shapes, and the death rate of the cultures.

It was found that no difference existed between the studied phenotypes of *H. pylori* 17874 and *H. pylori* Δcsg in a complex medium. The growth, conversion, and death rates were similar for both strains (Fig. 3.10 and Fig. 3.11). Growth occurred soon after inoculation but a lag phase was not observed, possibly due to the long time intervals between sampling. The cultures reached their maximum density after approximately 40 hours, at which time the number of coccoid cells began to increase. There was a proportional increase in coccoid cells as the number of viable cells decreased (Fig. 3.12). During this time, the OD of the culture also dropped due to the change in cell shape (results not shown). No viable cells were detected after 96 hours after inoculation.

3.3.4.2 Phenotypic analysis in defined medium

As there was no phenotypic difference between the wild type and the mutant *H. pylori* strains in complex medium, it was decided to investigate the phenotypes in a defined medium which would allow the omission of individual components. Defined media for *H. pylori* have been described by Reynolds and Penn (1994) and Nedenskov (1994). It was decided to use the

Figure 3.10 Growth of *H. pylori* strains 17874 and Δcsg in complex medium (TSB)

The growth of *H. pylori* strains 17874 and Δcsg over a period of five days is shown. The graph shows the growth rates of both strains to be the same. The two strains also become non-viable at the same rate.

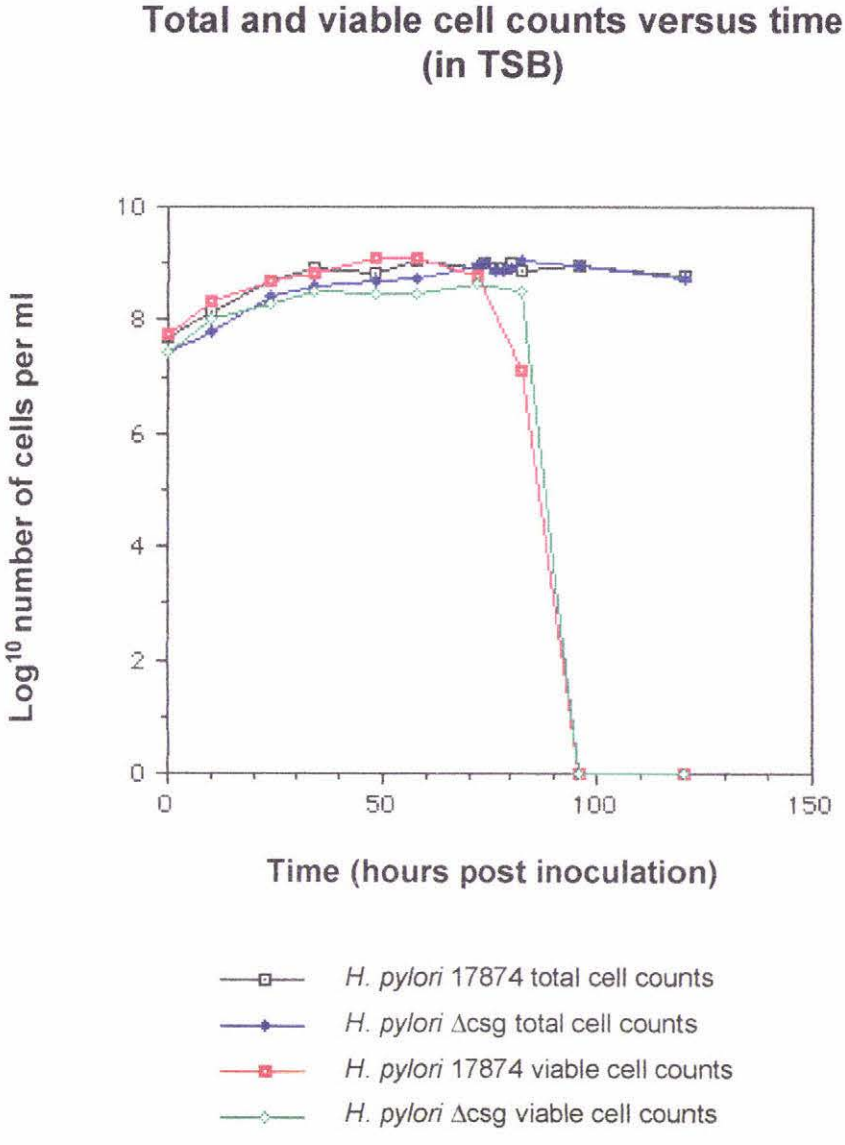

Total and viable cell counts versus time
(in TSB)
Log10 number of cells per ml
10
8
6
4
2
0
0
50
100
150
Time (hours post inoculation)
H. pylori 17874 total cell counts
H. pylori Δcsg total cell counts
H. pylori 17874 viable cell counts
H. pylori Δcsg viable cell counts

Figure 3.11 The conversion of rod to coccoid shape cells in complex medium (TSB)

The percentage of coccoid cells in cultures of *H. pylori* strains 17874 and Δcsg are shown.

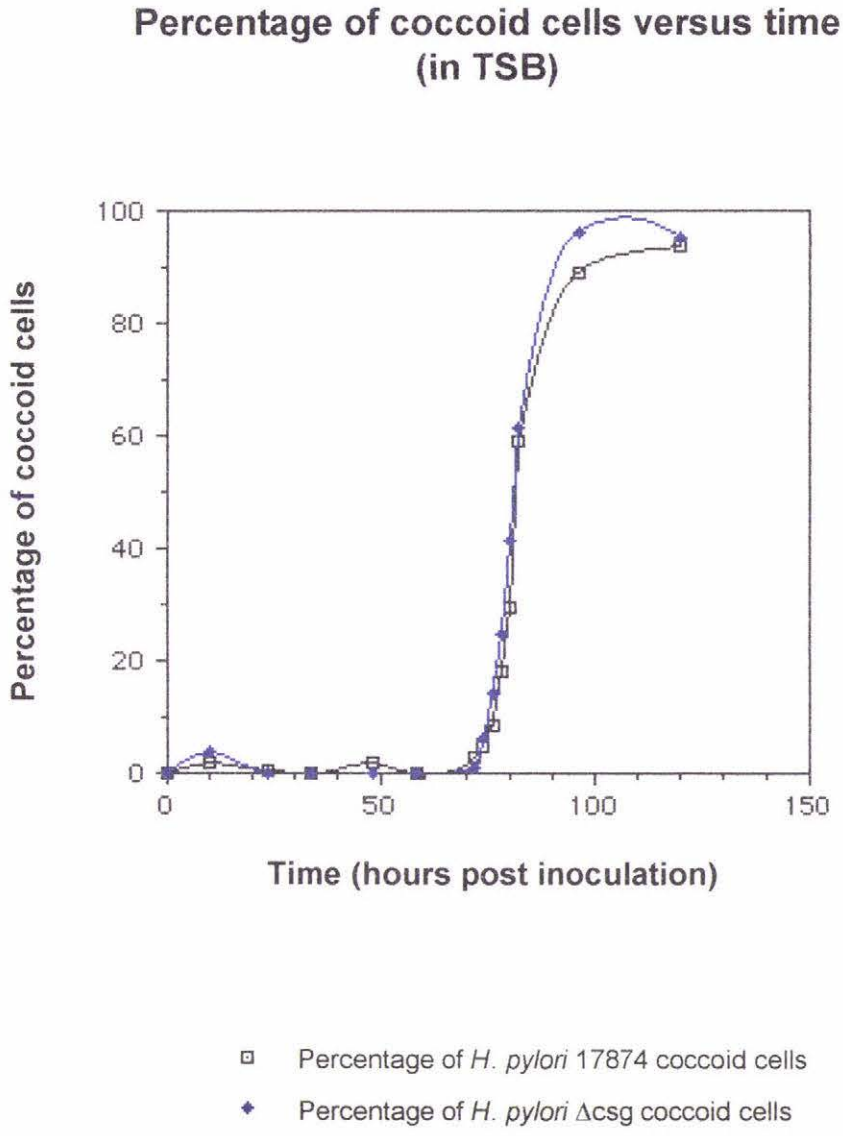
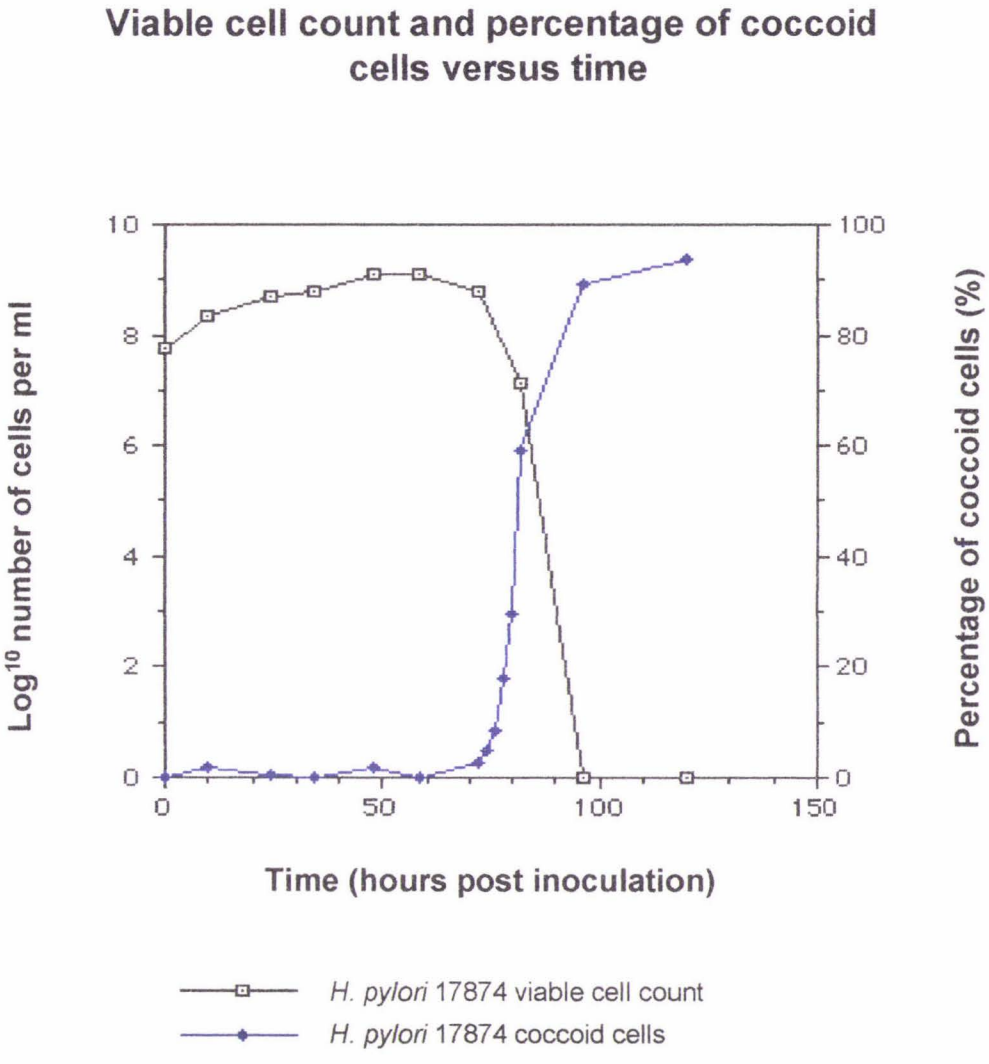

Percentage of coccoid cells versus time (in TSB)
Percentage of coccoid cells
100
80
60
40
20
0
0
50
100
150
Time (hours post inoculation)
Percentage of H. pylori 17874 coccoid cells
Percentage of H. pylori Δcsg coccoid cells

Figure 3.12 The relationship between the death and conversion rates of H. pylori strains 17874 and Δcsg in complex medium (TSB)

The graph shows an inverse relationship between the death and conversion rates of the two *H. pylori* strains, 17874 and Δcsg. The number of viable cells decreases before the percentage of coccoid cells present in the culture increases, indicating that helical cells can also be non-viable.


Viable cell count and percentage of coccoid cells versus time
Log10 number of cells per ml
Percentage of coccoid cells (%)
Time (hours post inoculation)
0
2
4
6
8
10
0
20
40
60
80
100
0
50
100
150
H. pylori 17874 viable cell count
H. pylori 17874 coccoid cells

medium described by Reynolds and Penn (1994) for this experiment due to the local availability of compounds.

The wild type and Δcsg strains were grown in the defined medium over a period of seven days. The most obvious difference between growth in the defined medium and that in a complex medium was the rate of growth. Where maximum density was achieved after 40 hours in complex medium, the maximum density in the defined medium was achieved after 72 hours for the wild type strain and between 100-120 hours for the Δcsg strain (Fig. 3.13). Total cell counts were not obtainable after the culture reached maximum density as the cells tended to clump together, due possibly to the components in the medium (Stark *et al*, 1997).

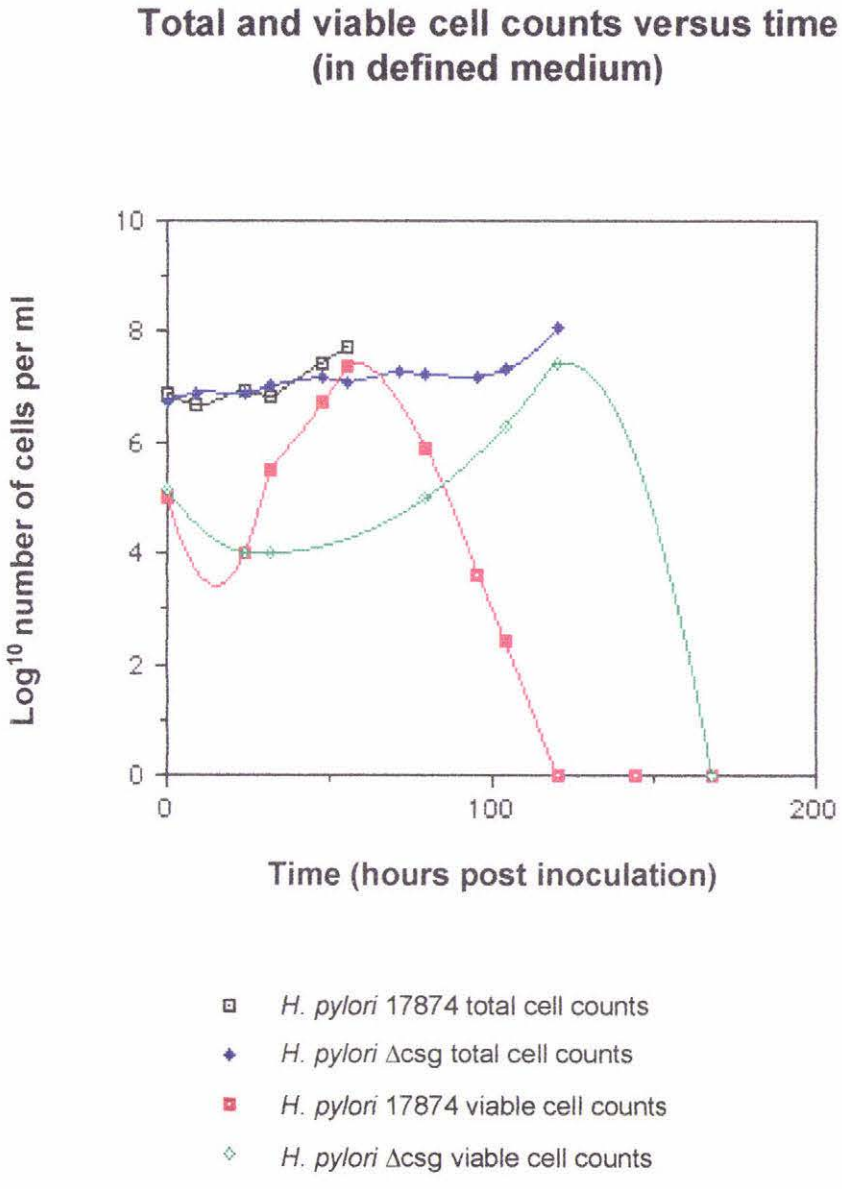
A significant lag phase was observed in the growth of the Δcsg strain. This generally lasted for 70-80 hours before significant growth began. The wild type strain, however, began to grow almost immediately after inoculation of the medium. The two strains grew at different rates, the wild type strain growing faster and, therefore, becoming non-viable earlier than the Δcsg culture. The death rates of the two strains remained similar.

The next growth experiment was performed in defined medium lacking in glucose. The aim of the experiment was to observe the growth after the removal of what is thought to be the major carbon source. The resulting growth curves, however, showed little, if any, difference between cultures grown with or without glucose (result not shown).

As a phenotypic difference was observed between the wild type and Δcsg strains when grown in a defined medium it was decided to study an additional *H. pylori* strain. *H. pylori* ΔglnP1

Figure 3.13 Growth of *H. pylori* strains 17874 and Δcsg in defined medium

Growth of strains 17874 and Δcsg over a period of seven days is shown.


Total and viable cell counts versus time
(in defined medium)
Log10 number of cells per ml
10
8
6
4
2
0
0
100
200
Time (hours post inoculation)
H. pylori 17874 total cell counts
H. pylori Δcsg total cell counts
H. pylori 17874 viable cell counts
H. pylori Δcsg viable cell counts

(Table 2.1) contains a non-functional *glnP1* gene. The Δcsg strain contains a non-functional *csg* gene but also a non-functional *glnP1* as the promoter region and transcription start point were removed during mutagenesis of *csg*. It was decided to compare the growth of the Δcsg and ΔglnP1 strains to determine whether or not the phenotypic difference was due to the non-functional *csg* gene or the *glnP1* gene.

The ΔglnP1 strain was grown in defined medium without glucose and either with or without glutamine in parallel with cultures of the Δcsg strain. Δcsg behaved differently in the two media, growing in medium with glutamine and becoming non-viable 50 hours after inoculation (Fig. 3.14), but in medium without glutamine, no growth occurred. However, the culture remained at a constant concentration of viable cells before becoming non-viable after 36 hours. In either medium, the ΔglnP1 strain did not grow and viable cell counts showed the culture to be non-viable within 24 hours after inoculation (Fig. 3.15). It was thought that glucose was necessary for growth of the ΔglnP1 strain and a subsequent growth experiment was performed in defined medium with glucose and either with or without glutamine. The growth curve (result not shown) gave similar results with the cultures being non-viable within 24 hours post inoculation.

It was decided to investigate whether or not the two mutant strains would grow in a medium without glucose and amino acids, and with peptides as a sole carbon source. This decision was made on the basis of the putative role of *E. coli* CstA in the transport of peptides. A medium was made based on the Reynolds and Penn (1994) defined medium. Glucose and all amino acids were omitted from the medium and replaced with tryptone (17g/l). The wild type, Δcsg, and ΔglnP1 strains were grown in this medium and the subsequent growth curve

Figure 3.14 ***H. pylori*** **Δcsg grown in defined medium without glucose and either with or without glutamine**

The growth of *H. pylori* Δcsg is shown over a period of six days in cultures lacking in glucose, and either with or without glutamine.

Total and viable cell counts versus time (in defined medium without glucose)

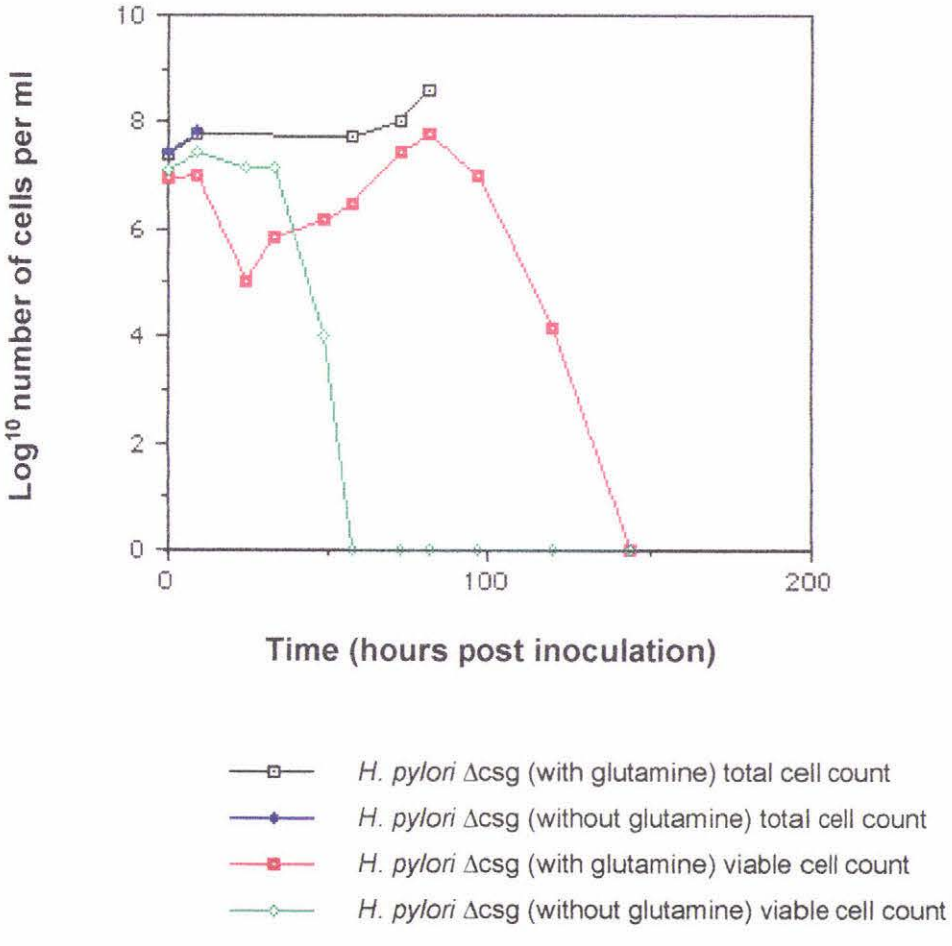
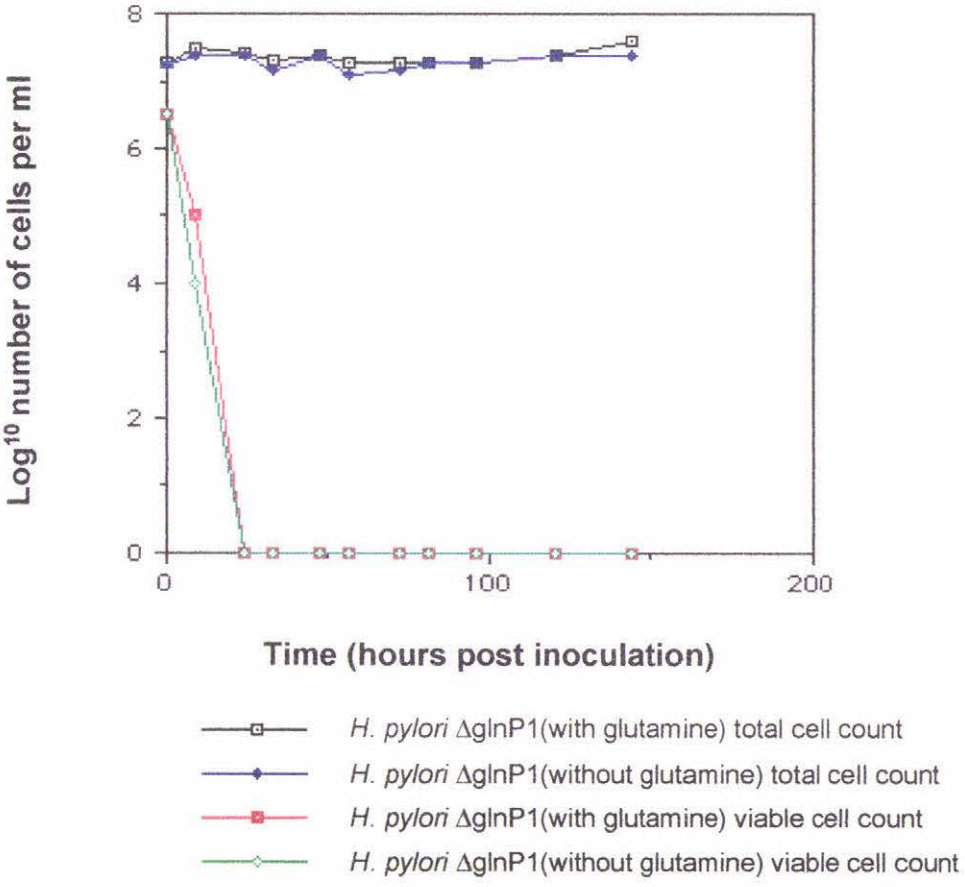


Figure 3.15 ***H. pylori*** **ΔglnP1 grown in defined medium without glucose and with or without glutamine**

The growth of *H. pylori* ΔglnP1 is shown over a period of six days in defined medium lacking glucose, and either lacking or containing glutamine

Total and viable cell counts versus time for ΔglnP1 (in defined medium)



(Fig. 3.16) showed no significant difference in the growth rate between the three strains. The growth rate also appeared to be the same as that observed in the complex medium, reaching maximum density after 40 hours.

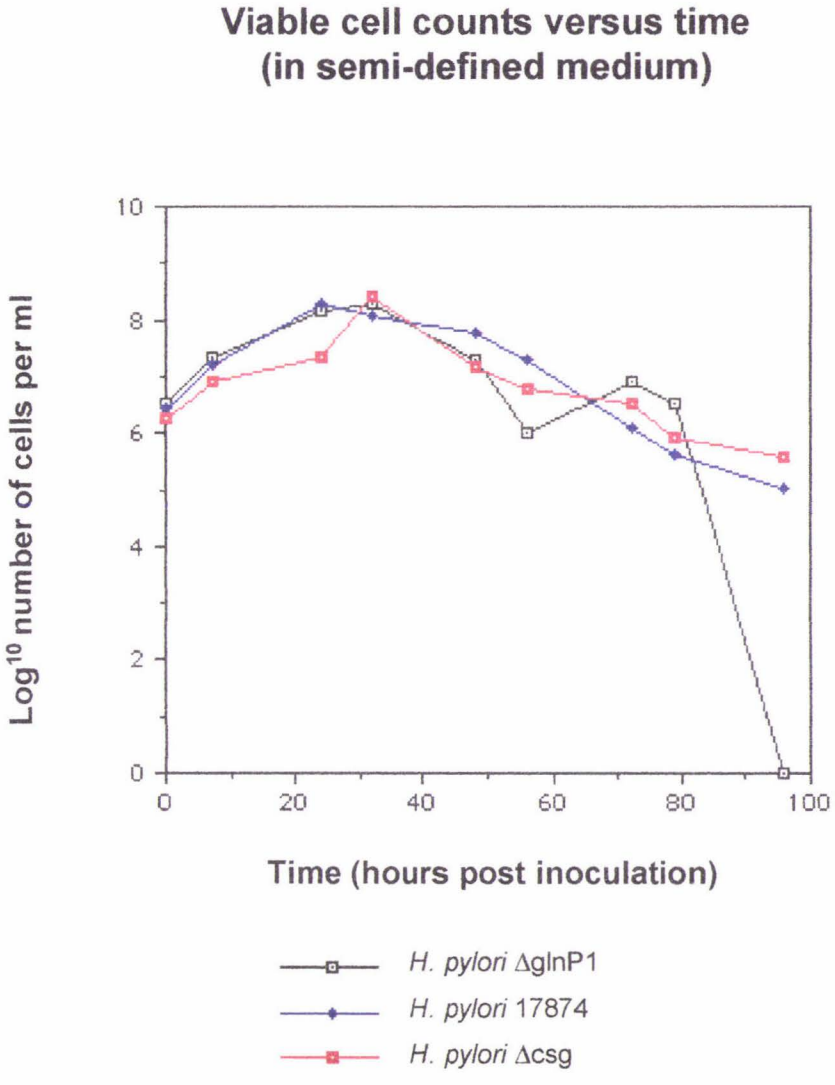
3.3.5 Determination of why the Δcsg and ΔglnP1 strains express different phenotypes with regard to the *glnP1* gene

The difference in growth of the ΔglnP1 and Δcsg strains was not expected as both strains possessed a non-functional *glnP1* gene. The Δcsg strain has lost the *glnP1* promoter and initial sequence of *glnP1* whereas the ΔglnP1 strain has an inserted CAT cassette in the 3' end of the gene. One possible explanation was that the *glnP2* homologue possesses sequence that is capable of acting as a promoter, which permits transcription of *glnP1* in the Δcsg strain, but not in the ΔglnP1 strain due to the presence of the CAT cassette in this region. It was theorised that the CAT cassette might be present in the opposite orientation of the operon, thereby disrupting any transcription from a possible promoter element of *glnP2*. As this CAT cassette was not present in the Δcsg strain, there was no inhibition of transcription from this hypothetical promoter.

To test the validity of this theory, diagnostic digests were performed to determine the orientation of the CAT cassette in the ΔglnP1 strain with respect to the glutamine permease operon. The CAT cassette, an 800bp cassette from the plasmid pRY109, possesses an asymmetrical *Hind*III site 600bp from the transcription start point. A *Hind*III digest was performed on the plasmid pHP041, which contained the mutation and was transformed into *H. pylori* 17874 to create the ΔglnP1 strain. The expected fragment sizes for this digest were

Figure 3.16 ***H. pylori*** **strains 17874, Δcsg, and ΔglnP1 grown in semi-defined medium**

The growth of the *H. pylori* strains 17874, Δcsg, and ΔglnP1 over a period of four days is shown.


Viable cell counts versus time
(in semi-defined medium)
Log10 number of cells per ml
10
8
6
4
2
0
0
20
40
60
80
100
Time (hours post inoculation)
H. pylori ΔglnP1
H. pylori 17874
H. pylori Δcsg

5kb, 1.8kb, 0.9kb, 0.7kb, and 0.3kb. The resulting digest is shown in Fig. 3.17. It was determined that the CAT cassette was, as suspected, in the opposite orientation to the glutamine permease operon.

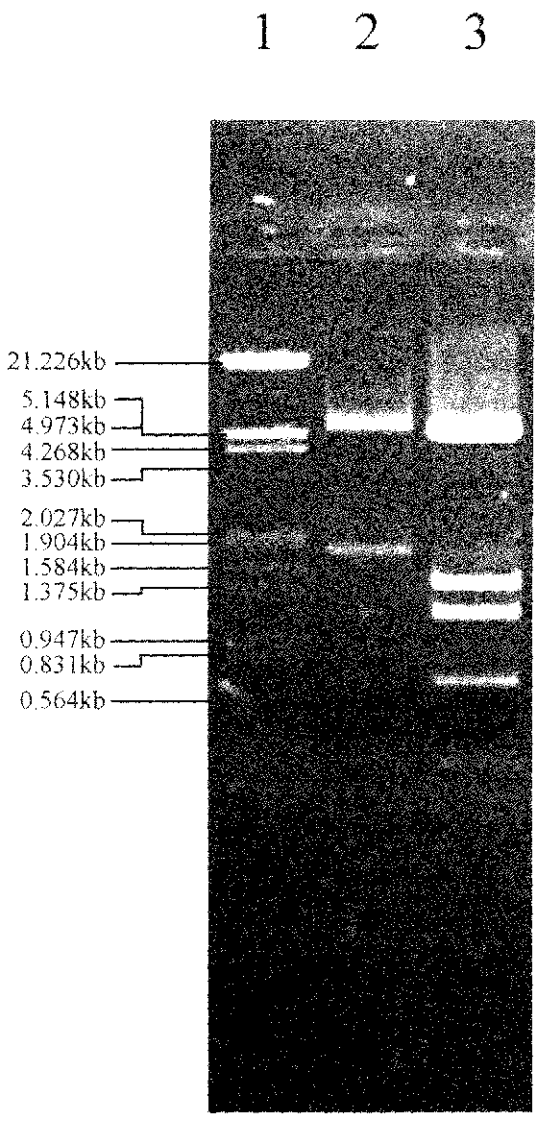
To determine whether or not a promoter element existed in *glnP1* for *glnP2*, RT-PCR analysis was used. *H. pylori* strains 17874, Δcsg, and ΔglnP1 were grown for 24 hours until at approximately mid-exponential phase, then pelleted and washed in PBS buffer. The pellets were then resuspended in CO_2-equilibrated defined medium and incubated for a further four hours. The cells were then pelleted and RNA was isolated using the TRIZOL reagent (Section 2.11.2). RT- PCR was then performed using primers C8 and C9, but yielded no results. Further limited attempts to isolate RNA and perform RT-PCR were unsuccessful. This may have been due to the small volumes of cell culture yielding little RNA or the possible degradation of RNA by RNAse contamination. Time constraints prevented further investigation into this problem.

3.3.6 Construction of a *H. pylori* Δcsg strain for colonisation studies

Plasmid pAB03 was transformed into *H. pylori* Sydney strain to enable colonisation studies in mice. The *H. pylori* Sydney strain is able to infect mice, unlike other *H. pylori* strains. By studying the mouse model, it would be possible to draw conclusions regarding the ability of an *H. pylori* strain possessing a non-functional *csg* gene to colonise the gastric mucosa of humans. The colonisation studies would be performed by Dr Wangxue Chen, of the Wakefield Gastroenterology Research Institute, Wellington, NZ. However, the results of these studies were not available at the time of publication of this thesis.

Figure 3.17 *Hind*III digest of pHP041 to determine the orientation of the CAT cassette

*Hind*III was used to determine the orientation of the CAT cassette in pHP041, which produced the mutation in *H. pylori* ΔglnP1. The CAT cassette contains an asymmetrical *Hind*III site. The photograph also shows the digestion of pHP040, which pHP041 is based on. Lane 1: λ*Eco*RI/*Hind*III ladder, Lane 2: pHP040 digested with *Hind*III, Lane 3: pHP041 digested with *Hind*III.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5.148kb
4.973kb
4.268kb
3.530kb
2.027kb
1.904kb
1.584kb
1.375kb
0.947kb
0.831kb
0.564kb

3.4 Analysis of the expression of *csg*

3.4.1 Isolation and quantification of RNA to study expression of *csg*

RNA was isolated and quantified to study the expression of *csg* over a period of time as the carbon source was diminished. As samples were collected twice daily, a quick and easy method was required to isolate the RNA. The TRIZOL reagent (Life Technologies) was chosen for this as the isolation process takes under two hours.

H. pylori was grown in 50mls of TSB and 10ml samples were removed morning and night for 2.5 days. The RNA was isolated as described in Section 2.11.2. 5µl of each of the five samples were then analysed by gel electrophoresis to confirm the presence of RNA. The remainder of the RNA samples were supplemented with Vanadyl Ribonuclease Complex I (VRC) to inhibit the action of RNAse, and stored at -80°C.

A *csg* probe was designed and produced by a PCR amplification reaction using two existing sequencing primers, CSG2 and C16 (Table 2.2). The PCR product was gel purified and stored at -80°C.

3.4.1.1 Northern blotting of csg *RNA*

The RNA samples were treated with RNAse-free, DNAseI to remove any residual DNA from the samples. The samples were then size fractionated by gel electrophoresis and the gel

prepared for blotting (Section 2.12.1). The resulting autorad showed no expression of *csg*. The blot was reprobed with a probe designed to bind to a gene which continually expresses a 26kDa protein and is a positive control for *H. pylori* RNA analysis. The resulting autorad showed no bands for this positive marker so it was thought that the RNA may have been degraded by accidental introduction of RNAse.

Isolation of RNA from *H. pylori* was attempted a further two times but with no success. All solutions were remade to ensure that RNAse was not unintentionally introduced, but this did not alter the outcome. It was then thought that not enough RNA was being isolated from the small sample volume. As other RNA isolation methods involved a significant amount of time or bacteria, and that eventually samples would be taken every two hours, it was decided to seek an alternative strategy for studying *csg* expression, which would have the added advantage of allowing quantitative measurement to be made.

3.4.2 Construction of a CAT expression plasmid

An alternative strategy to RNA analysis to study the expression of *csg* was to construct an expression plasmid with a reporter gene cloned into a site downstream of *csg*. This plasmid could then be transformed into *H. pylori* and the reporter gene incorporated into the genome by homologous recombination.

A further consideration was that the reporter gene was to be placed under the promoter of a gene involved in, and possibly only expressed during carbon starvation, so it could not necessarily be selected for after transformation. For this reason, it was necessary to ligate the

reporter gene to an antibiotic marker prior to cloning into the plasmid. The strategy for the construction of the new plasmid is shown in Fig. 3.18.

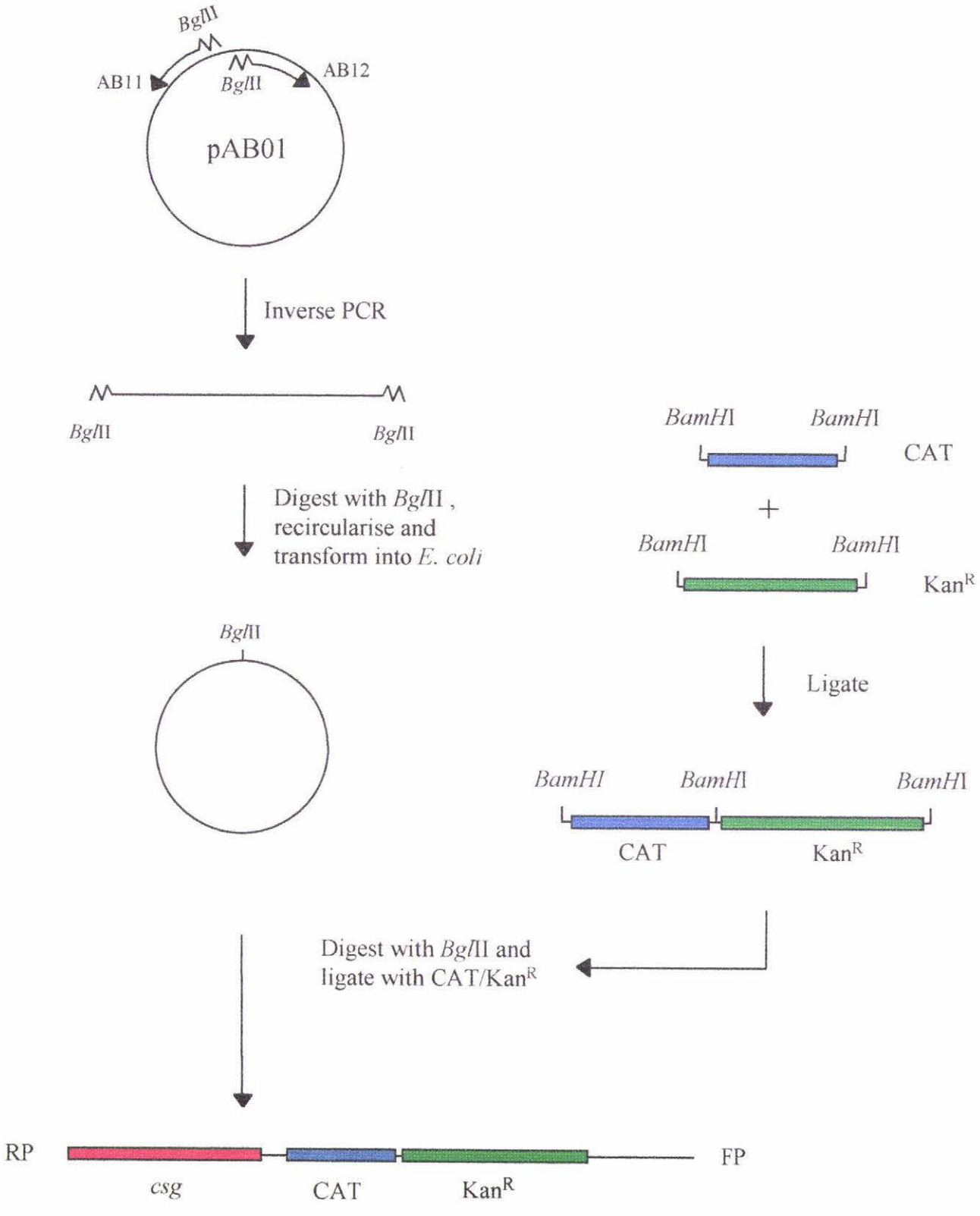
The promoterless chloramphenicol acetyl transferase (CAT) gene was chosen as the reporter gene for this experiment. This gene has been used successfully to study the environmental regulation of the expression of Campylobacter flagellin genes (Alm *et al*, 1993). The CAT gene was excised from pCM4 (Table 2.1) by cleaving with the restriction endonuclease *Bam*HI. A kanamycin resistance gene (Kan^R) was chosen as the selectable marker and excised from pILL600 (Table 2.1) also by cleaving with *Bam*HI. The two purified DNA fragments were stored at -20°C.

Analysis of the sequence downstream of *csg* on the plasmid pAB01 revealed no unique restriction enzyme recognition sequences. It was decided to engineer a recognition site for *Bgl*II, which was not already present in either the insert or plasmid vector, 10bp downstream of the *csg* stop codon to disrupt the suspected transcription terminator and allow the insertion of the CAT gene. Oligonucleotide primers were designed for an inverse PCR amplification, with 5' tails containing *Bgl*II recognition sequences. The resulting PCR product was gel purified and stored at -20°C until required.

The purified PCR product was self ligated and then transformed into *E. coli* ER2206. Five colonies were screened using the quick alkaline lysis preparation (Section 2.5.2). All five transformants were the expected size and to confirm a successful ligation, were digested with *Bgl*II. Furthermore, a sample of an alkaline lysis/PEG precipitation preparation was

Figure 3.18 Strategy for the construction of the CAT expression plasmid

The strategy for the construction of the CAT expression plasmid is shown. This initial strategy differs to that finally used in that the CAT and Kan^R cassettes were not ligated, but instead used in three-way ligations with the vector DNA.


BglII
AB11
BglII
AB12
pAB01
Inverse PCR
BglII
BglII
Digest with BglII ,
recircularise and
transform into E. coli
BglII
BamHI
BamHI
CAT
+
BamHI
BamHI
KanR
Ligate
BamHI
BamHI
BamHI
CAT
KanR
Digest with BglII and
ligate with CAT/KanR
RP
FP
csg
CAT
KanR

sequenced using primer AB07 (Table 2.2) to confirm the presence of the *Bgl*II site; the resulting sequence clearly showing the presence of the *Bgl*II site. The *Bgl*II digested plasmid DNA was then gel purified and stored at -20°C. The plasmid was named pAB04 (Appendix A.7).

Various ligations were carried out in an attempt to clone the CAT and Kan^R cassettes into the *Bgl*II site of pAB04. These included ligating the CAT and Kan^R cassettes and isolating the 2.2kb product that was cloned into pAB04, repeating this without isolating the 2.2kb fragment, and performing ligations in the gel slice to avoid loss of DNA. All of these attempts proved unsuccessful.

Three way ligations were then attempted. This required three stages of screening to identify the desired plasmid. The first stage was the selection of transformants that grew on L agar plates supplemented with kanamycin (25μg/ml). Transformants were then screened for the desired size of 7.4kb. pAB03 was also 7.4kb in size and was used for a standard marker during gel electrophoresis. Those transformants that were found to be 7.4kb were then subject to a diagnostic digestion with *Eco*RI. *Eco*RI cuts the plasmid five times, resulting in four fragments of DNA. The expected sizes are shown in Table 3.1. All 44 plasmids from 10 ligations that were screened in this third stage, were shown to contain Kan^R adjacent *csg*, rather than the CAT gene as desired.

Ligation attempts then returned to two step ligations. The CAT and Kan^R genes were ligated and after three hours, linear pAB04 was added to the reaction and left to incubate overnight.

Table 3.1 Expected sizes from diagnostic digestion of Kan^R transformants

Orientation	Expected sizes of fragments
Correct orientation (csg:CAT:Kan^R)	3.5kb 2.0kb 1.4kb 0.5kb
Incorrect orientation (csg:TAC:Kan^R)	3.5kb 2.3kb 1.8kb 0.2kb
Incorrect orientation (csg:Kan^R:TAC)	3.7kb 1.8kb 1.4kb 0.5kb
Incorrect orientation (csg:Kan^R:CAT)	4.0kb 1.8kb 1.4kb 0.2kb

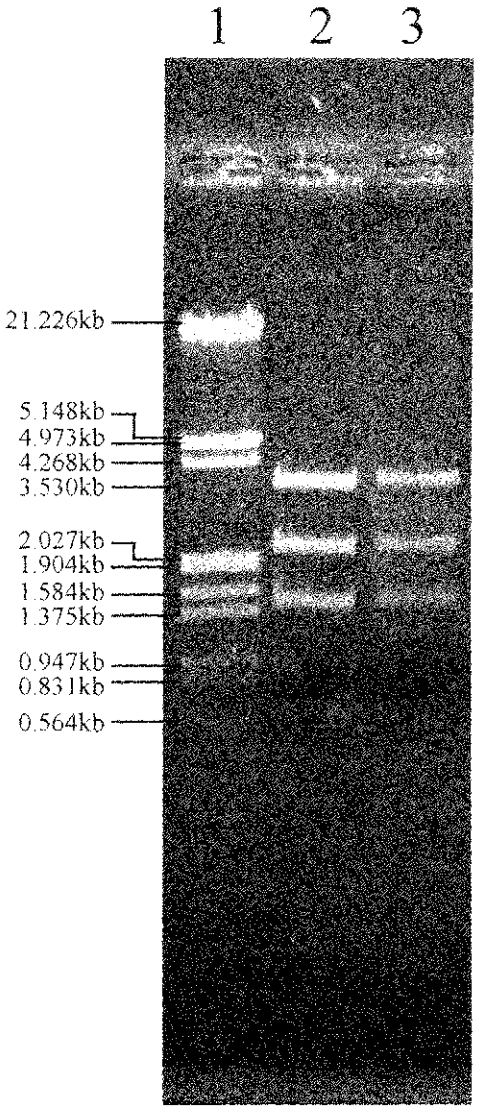
No transformants were produced by this method, although a gel photograph clearly showed that ligation products of the expected sizes were formed.

PCR amplification of the CAT and Kan^R cassettes after ligation was then attempted. Primers KAN1 and CAT1 (Table 2.2) were designed with *Bgl*II tails, allowing the PCR product, with the cassettes in the correct orientation, to be cloned into the *Bgl*II site of pAB04. A PCR product was successfully produced and gel purified, but this method was abandoned, as renewed attempts using the three way ligation method were successful.

Attention had turned back to three-way ligation, as this was the only method that had produced any plasmids with both the CAT and Kan^R cassettes, even if in the wrong orientation. It was decided to use a greater amount of DNA than previously used. The ligation reaction contained approximately 100ng each of the CAT cassette and the Kanamycin resistance marker, and 30ng of the pAB04 vector. The ligation reaction was shown to be successful through gel electrophoresis of samples taken before and after incubation with T4 DNA ligase. This revealed that individual components of the ligation were reduced in concentration, indicating that ligation had occurred. The reaction was subsequently transformed into *E. coli* ER2206, resulting in 25 transformants that were capable of growth in the presence of kanamycin. Twenty of these colonies were screened for the desired size of 7.4kb, of which five were retained for diagnostic digestion. The five isolates were digested with *Eco*RI to produce the four patterns shown in Table 3.1. Two isolates were found to possess the desired restriction pattern (Fig. 3.19). To confirm that these plasmids possessed the genes in the desired orientation, a double digest using *Eco*RI and *Bam*HI was performed. The expected sizes were 2.6kb, 1.4kb, 1.2kb, 0.9kb, 0.8kb, and 0.5kb. Both plasmids possessed the desired orientation (Fig. 3.20), and to double-check, a

Figure 3.19 Diagnostic digest of *E. coli* transformants

Two 7.4kb plasmids were digested with *Eco*RI to determine the orientation of the CAT and Kan^R cassettes. Lane 1: λ*Eco*RI/*Hind*III ladder, Lane 2: plasmid isolate #5, Lane 3: plasmid isolate #17.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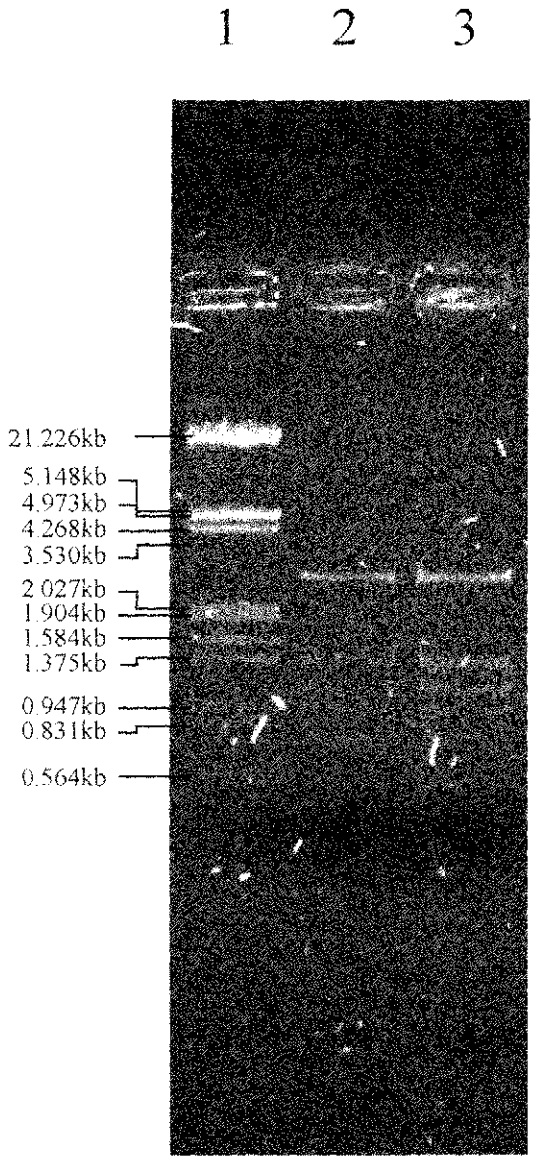
21.226kb
5.148kb
4.973kb
4.268kb
3.530kb
2.027kb
1.904kb
1.584kb
1.375kb
0.947kb
0.831kb
0.564kb

Figure 3.20 Double digest of *E. coli* transformants

Plasmids #5 and #17 were digested with *Eco*RI and *Bam*HI to confirm the orientation of the CAT and Kan^R cassettes. The fragment pattern generated was more conclusive than that of a digest with *Eco*RI. Lane 1: λ*Eco*RI/*Hind*III, Lane 2: plasmid isolate #5, Lane 3: plasmid isolate #17.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21.226kb
5.148kb
4.973kb
4.268kb
3.530kb
2.027kb
1.904kb
1.584kb
1.375kb
0.947kb
0.831kb
0.564kb

PCR amplification reaction was performed. The CAT1 and reverse primers were used to amplify the plasmid DNA which was expected to yield a 3.0kb fragment. KAN1 was also used in two reactions - one with reverse primer and one with forward primer to orientate the gene. This would produce fragments of 2.7kb or 4.0kb, respectively. The PCR results confirmed the presence and position of the CAT cassette in the DNA construct (results not shown). The presence of a 4.0kb fragment in the KAN1-forward primer reaction indicated that the kanamycin resistance cassette was transcribed in the same direction as the *csg* and CAT genes. The new plasmid was named pAB05 (Appendix A.8).

pAB05 was transformed into *H. pylori* using natural competence (Section 2.8.5). Recombination of the plasmid into the *H. pylori* chromosome was selected for by the ability of transformants to grow on kanamycin supplemented CBA plates. A representative of the successful transformants was single colony purified and chromosomal DNA was isolated. A PCR reaction was performed on the DNA using primers CAT1 and AB03, and expected to yield a fragment of 3.0kb. The resulting PCR product, of the expected size, confirmed both the presence and orientation of the CAT gene in the *H. pylori* chromosome. The transformant was put into glycerol stocks for storage at -80°C. Further experiments with this strain were not conducted due to time constraints.

4.0 Discussion

4.1 Sequencing of *H. pylori* 17874 *csg*

The sequencing *H. pylori* 17874 *csg* and *glnP1* and the subsequent comparison with the sequence of the H. pylori 26695 *cstA* produced two significant results. These are discussed below.

4.1.1 *csg* may have been acquired by *H. pylori* from another organism

The *H. pylori* 17874 *csg* gene has been shown to have a high G+C content in comparison with that of the genome. As discussed briefly in the results section, the G+C content of *H. pylori* 17874 has been estimated to be 35.6% (Béji *et al*, 1988), while the *csg* sequence exhibited a G+C content of 44%. Such a difference in G+C content is usually associated with insertion sequences or genes that have been acquired from a different organism. Example of these are the five regions present in the *H. pylori* 26695 genome , which possess a G+C content that differs significantly from that of the genome. Two of these regions contain insertion sequences, one of which also contains orthologues of *virB4/ptl*, the product of which is involved in the transfer of the oncogenic T-DNA in *Agrobacterium* and the secretion of the pertussis toxin by *Bordetella pertussis* (Tomb *et al*, 1997). This significant difference in G+C content between the genome and *csg* of *H. pylori* 17874 may indicate that the *csg* gene was acquired from another bacterium, which may have had a G+C content of approximately 44%.

4.1.2 *H. pylori* 17874 exhibits sequence diversity to *H. pylori* 26695

The *csg* gene of *H. pylori* 17874 shows considerable sequence diversity to that of *H. pylori* 26695, with *H. pylori* 17874 *csg* demonstrating 96.4% identity to the *csg* of *H. pylori* 26695. This is, however, a well documented characteristic of *H. pylori*. The diversity of nucleotide sequence of *H. pylori* isolates has enabled typing of strains through the use of restriction endonucleases (Fujimoto *et al*, 1994; Majewski *et al*, 1988; Labigne, 1997). As a result of this sequence diversity, changes in amino acid residues between strains are also to be expected.

Although sequence diversity is an accepted and defining characteristic of *H. pylori*, *csg* may have been acquired from another organism, which may or may not have such diversity. However, sequence comparison of the *H. pylori* 17874 *csg* gene and that of *H. pylori* 26695 show *csg* to exhibit sequence diversity. This indicates that *csg* was acquired by *H. pylori* before strain diversity became established.

4.2 Linkage between *csg* and *glnP1*

Evidence obtained through sequencing of the *csg* and *glnP1* promoter regions (Section 3.2) lead to the investigation of possible linkage between the two genes. The putative CRP binding site, which binds cAMP, a possible regulator of *csg*, was found in the 5' sequence of the *glnP1* gene. PCR amplification of the *csg:glnP1* intergenic region showed ten out of ten *H. pylori* strains to possess a region of the same size. *H. pylori* 26695 was also found, through analysis of the sequence, to have almost the same size intergenic region. It was concluded on the basis

of this evidence that the this gene arrangement is conserved among *H. pylori* strains. As *glnP1* is part of the glutamine permease operon, it can be further concluded that *csg* is linked to this entire operon.

Jaing *et al* (1996) have shown *H. pylori* strains to be diverse in regard to gene order. For eleven strains, nine of which are clinical isolates, to possess an identical gene order with regards to *csg* and *glnP1* was deemed significant. PCR amplification of a *H. mustelae* strain, however, has demonstrated that this linkage is possibly specific to *H. pylori* strains.

The linkage of *csg* and *glnP1* in *H. pylori* strains may be important for cell metabolism. cAMP may be a regulator of *csg*, but may also act as an inhibitor of *glnP1*. It is possible that the two gene products are also mutually exclusive and are ultimately regulated by the environment. When a carbon source is readily available, active growth take place. During this time, *csg* may not be expressed or is expressed at low basal levels, as intracellular concentrations of the regulator, cAMP, are low. At the same time, the glutamine permease operon is actively transporting glutamine into the cell to provide a source of glutamine, nitrogen, or precursors for pyrimidine and protoheme biosynthesis (Tomb *et al*, 1997). When the carbon source becomes exhausted, cell growth reaches a stationary phase and the intracellular concentration of ppGpp is increased. This, in turn, leads to the conversion of AMP into cAMP which then acts as a regulator for genes involved in the carbon starvation stringent response. In binding to the putative CRP binding site of *csg*, the *glnP1* gene would no longer able to be transcribed and would, in effect, be inhibited. At this stage of cell life, there is little need for the transportation of glutamine into the cell. As active growth is no longer occurring, the break down of glutamate into glutamine may be able to supply the cellular requirements.

It is unknown why this gene arrangement is limited to *H. pylori*. It is possible that in the hostile environment of the stomach, it is to the bacterium's advantage to have a mechanism, which allowed fast switching from one cellular metabolic process to another during times of stress. However, this would also be expected of other *Helicobacter* species.

4.3 Mutagenesis of *csg* and phenotypic analysis of the Δcsg strain

4.3.1 Problems encountered while constructing pAB02

Several problems arose while attempting to mutagenise the *csg* gene present on pHP040. The first of these problems was producing sufficient quantities of a composite fragment of arm1 and arm2. Arm1 and arm2 were used as DNA template in a PCR amplification reaction with primers that would amplify a composite fragment only. The two components annealed at the *Bgl*II overhangs during the annealing phase of the PCR cycles. The resultant PCR fragment was then used as template DNA for subsequent cycles. While this strategy worked, the yield of the fragment was low, particularly after gel purification.

The second problem encountered was the inability to clone the composite fragment into pGEM-T. This was thought to be due to a problem with the pGEM-T kit rather than human error as other members of the laboratory were also unable to clone different fragments into the vector.

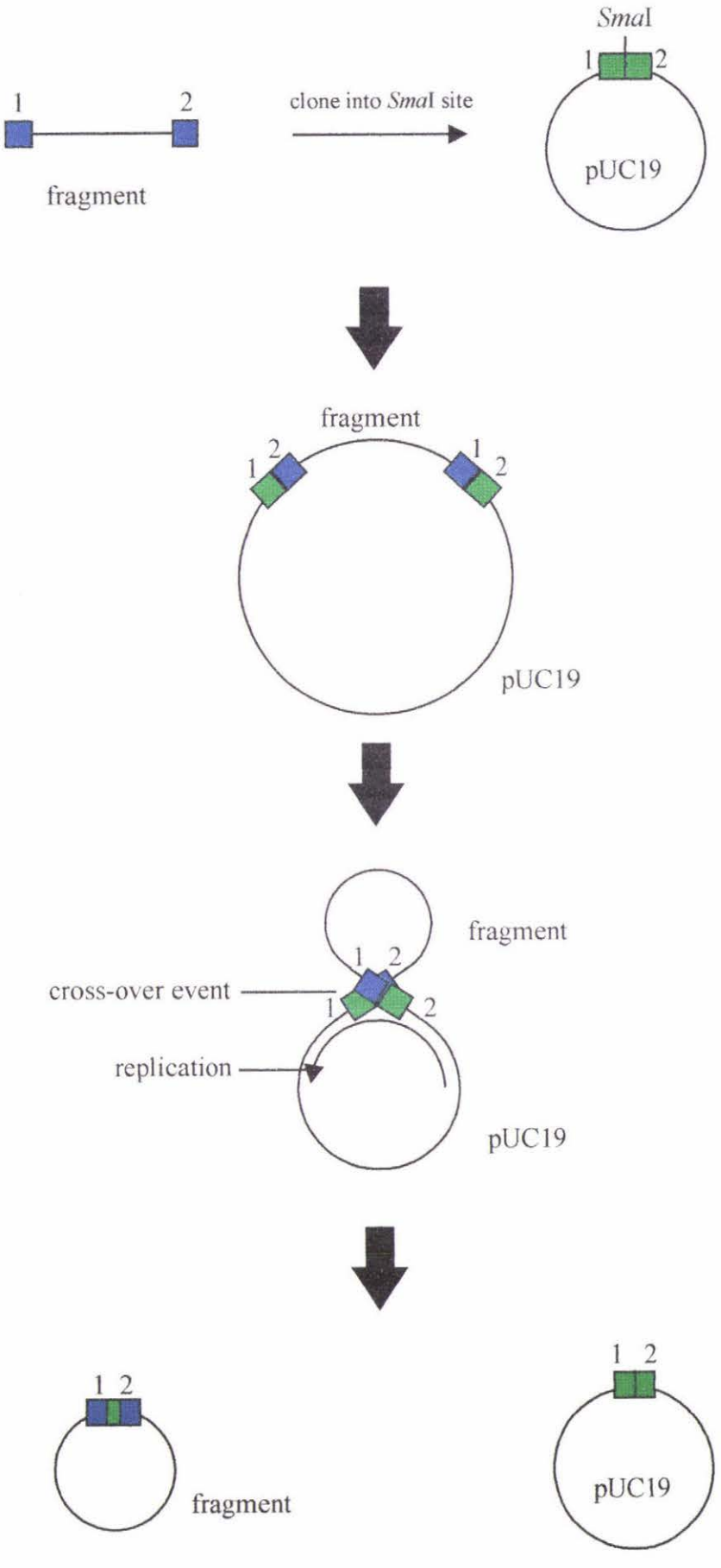
The decision to make a blunt-ended fragment and clone it into pUC19 cleaved with *Sma*I solved the first problem of obtaining sufficient quantities of the composite PCR fragment. The

use of *Pwo* DNA polymerase, which produced a blunt-ended fragment, yielded high quantities of PCR product, with a good concentration remaining after gel purification. However, cloning of this fragment into pUC19 produced no transformants containing both vector and insert. As blue/white colour selection was available, white colonies were screened but were found to have a small deletion rather than the desired insert. This was most probably due to the inclusion of the pBK-CMV multiple cloning site (MCS) in the PCR fragment, as forward and reverse primers were used in the amplification reaction. When this fragment was cloned into the MCS of pUC19, two copies of related MCS would then exist in the plasmid, which may make the insert unstable or allowed fragment excision by means of a cross-over event. The excision of the insert may result in the white colonies that were subsequently screened for the presence of the insert. As the insert and vector MCS were not identical, misalignment during a cross-over event could result in the inclusion of part of the insert MCS in the vector, or exclusion of part of the vector MCS. This, in turn, may cause a frameshift within the vector MCS, disrupting the transcription of β-galactosidase. This would have resulted in the formation of a white colour by the transformant colonies and account for the apparent deletion in pUC19 observed in the screened transformants However, this excision event would be dependent on the orientation of the insert within the vector as shown in Fig. 4.1. As a result of this instability, the cloning strategy had to be revised. The new strategy was to use primers internal to the insert of pHP040 so that the composite fragment would possess no duplications of sequences present on pUC19.

After changing the polymerase and primers used to produce the fragment, and the vector into which the fragment was cloned, pAB02 was constructed. The remainder of the mutagenesis strategy was completed without any problems, resulting in a new *H. pylori* strain to aid in the study of *csg*.

Figure 4.1 Possible excision of the composite PCR fragment from pUC19

The composite PCR fragment contains the MCS from the pBK-CMV (represented by the blue squares) backbone of pHP040, resulting in two similar copies of the MSC once the fragment is cloned into the MCS of pUC19 (represented by the green squares). If the insert is cloned into pUC19 in the orientation shown, *E. coli* could excise the fragment by forming a figure eight, allowing the MCS to align, and resulting in a cross-over event. Replication would be carried out through this cross-over, resulting in the reverted pUC19 and a circular fragment, which, possessing no origin of replication, is lost. The pUC19 plasmid may also contain a small fragment of the pBK-CMV MCS which would result in the disruption of β-galactosidase transcription. Such plasmids would cause the transformed colonies to appear white.


SmaI
1
2
clone into SmaI site
fragment
pUC19
fragment
pUC19
fragment
cross-over event
replication
pUC19
fragment
pUC19

4.3.2 The phenotype of the *H. pylori* Δcsg strain and role of Csg during carbon starvation

Phenotypic analysis of *H. pylori* Δcsg has been unable to determine an exact role for Csg. There appears to be little difference between the wild type strain and the Δcsg strain in growth, with the exception of that in the defined medium. Of more importance, there appears to be no difference in the viability of cells after the carbon source is exhausted. While this may suggest that the *csg* gene is not expressed during in carbon starvation at stationary phase, the carbon source present in the defined medium may be less than desirable for adequate *H. pylori* growth, such that Csg is required during exponential growth to supplement the substrates used as a carbon source. This would account for the long lag phase and slow growth rate of Δcsg in defined medium. In a complex medium, the two strains have the same growth rate, possibly because there are sufficient carbon concentrations present to support vigorous growth. As a result, Csg is not required to scavenge for substrates.

The similarities in results in this study using complex medium, and those of Schultz and Matin (1991) in the study of *E. coli cstA*, suggest that *csg* may have a similar role in the carbon starvation response as *cstA*. That is, a role in scavenging to increase the carbon sources available to the cell during carbon starvation.

4.3.2.1 Carbon sources available to *H. pylori*

The major carbon source of *H. pylori* has been thought to be glucose and possibly amino acids (Reynolds and Penn, 1994; Nedenskov, 1994; Mendz and Hazell, 1994; Stark *et al*, 1997).

The experiments performed during this study have shown that *H. pylori* can grow without glucose but with amino acids as a sole carbon source. More importantly, *H. pylori* has been shown to grow without glucose or amino acids, but with peptides as a sole carbon source. In the natural environment of the stomach, peptides are abundant and are easily broken down to supplement the cell with the required amino acids. Evidence for this was demonstrated by the inability of the Δ*glnP1* strain to grow in the defined medium, but the ability to grow in the semi-defined medium which contained no glucose or amino acids but peptides instead. In a medium lacking peptides, the inability to import glutamine into the cell proved to be fatal. However, wild type strains are able to grow in defined medium lacking both glutamine and peptides. If no glutamine was present in the medium to be imported into the cell, then a phenotype similar to that of the ΔglnP1 strain would be expected. A possible explanation for this apparent contradiction is that the glutamine permease transporter may be capable of transporting additional amino acids required for growth, and not exclusively glutamine. It would be possible to study this further in the future by performing transport assays.

4.3.2.2 Different phenotypes for Δcsg and ΔglnP1 strains

H. pylori Δ*glnP1* was used in this study to determine if the phenotypic differences of Δcsg compared with the wild type strain were due to the mutation of the *csg* or *glnP1* gene. Δ*glnP1* contains the CAT cassette from pRY109 inserted into the *glnP1* gene, whereas Δcsg contains an *glnP1* gene without the promoter region or the first 50bp of sequence. It was expected that the two different mutations would result in the same phenotype. However, the Δ*glnP1* strain did not grow in the defined medium whereas the Δcsg strain grew slowly. Both strains grew equally well in medium with peptides as a carbon source, whether it was the

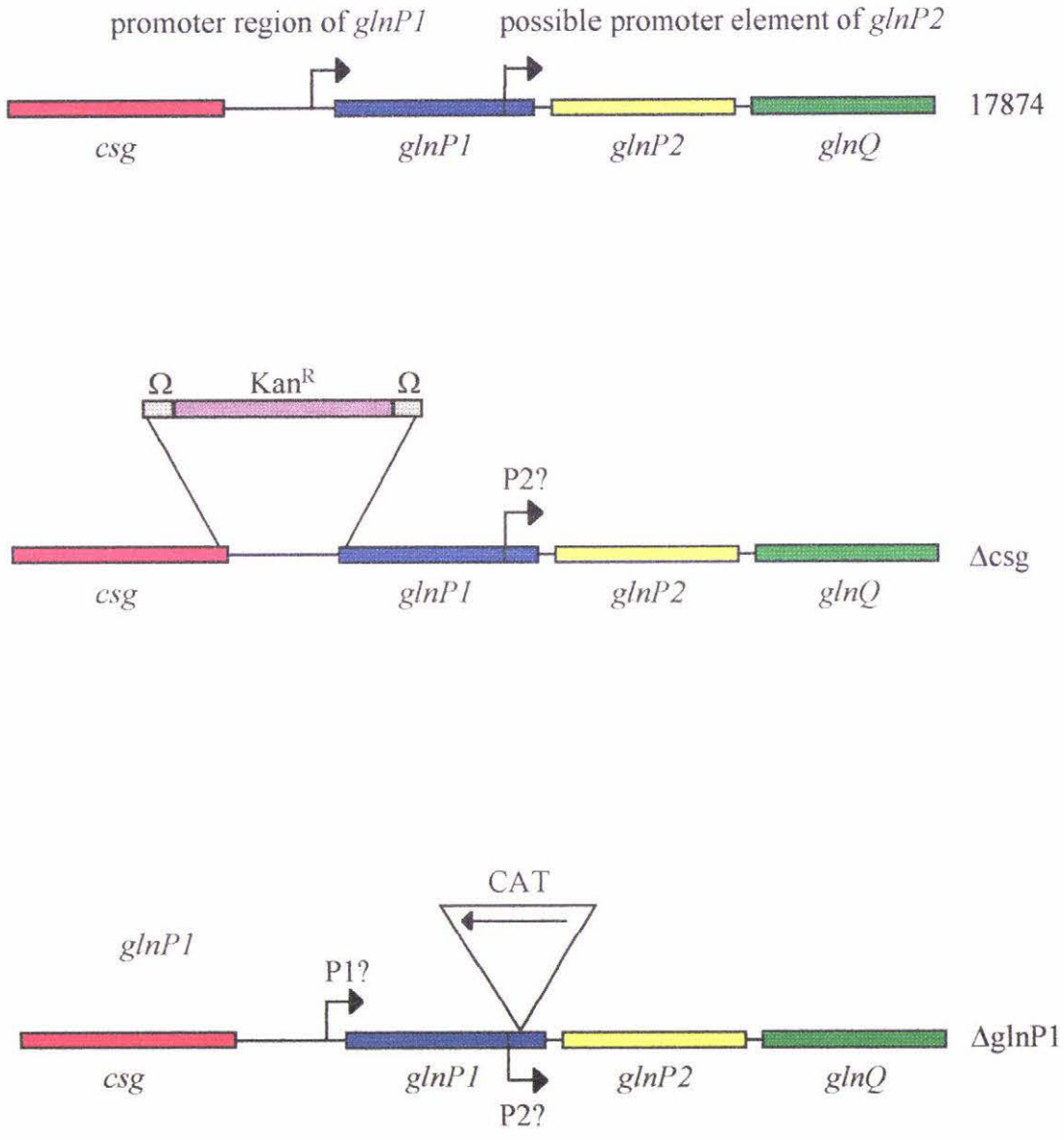
complex or semi-defined medium. A possible explanation for this occurrence is that a sequence capable of acting as a promoter exists in the *glnP1* gene, which allows transcription of *glnP2* (Fig. 4.2). When the promoter of the *glnP1* gene in the Δcsg strain is absent, transcription of *glnP2* would still possible. As there are two *E. coli glnP* homologues within the *H. pylori* glutamine permease operon, transcription of the second, functional homologue would result in no change in the phenotype, as seems to be observed in growth of the Δcsg strain. The insertion of the CAT cassette in *glnP1* of the Δ*glnP1* strain, however, may have disrupted this promoter element as the CAT cassette is transcribed in the opposite direction to the glutamine permease operon, therefore, disrupting the transcription of the *glnP2/Q* genes downstream of *glnP1*. This would affect the phenotype, resulting in a non-functional glutamine permease transporter, as is observed in the growth of the Δ*glnP1* strain.

RT-PCR was attempted to determine whether or not *glnP2/Q* are transcribed in Δcsg and ΔglnP1, but produced no amplification product, even for the wild type strain. It was thought that this was due to degradation of RNA or that a 10ml liquid culture does not provide enough cells to from which to isolate RNA.

However, if the theory that a promoter element exists for *glnP2* is correct, there are implications for the co-regulation of *csg* and the glutamine permease operon. Although the fact that *csg* and the glutamine operon are linked is not changed, the two gene products may not be mutually exclusive as previously thought. The rational behind the theory of Csg and the glutamine permease operon gene products being mutually exclusive was that the regulator of *csg*, cAMP, bound to the CRP binding site within the 5' sequence of *glnP1*, thereby inhibiting the transcription of this gene and, as was thought, the *glnP2* and *glnQ* genes. However, if a

Figure 4.2 A possible promoter element of *glnP2* may allow transcription of the glutamine permease operon when the *glnP1* gene is non-functional

The current level of understanding is that *glnP1* possesses a putative σ^{70} promoter which transcribes *glnP1*, *glnP2*, and *glnQ*. As a result, it was expected that removing the promoter of *glnP1* while constructing the Δcsg strain would result in the same phenotype as the ΔglnP1 strain. This was proved to be incorrect. A subsequent theory was put forward - that *glnP2* possesses a promoter element that allows transcription of *glnP2/Q*. This figure shows the putative position of the *glnP1* and *gln2P* promoters. When the *glnP1* promoter of Δcsg is removed, the *glnP2* promoter is able to transcribe *glnP2/Q*. In the ΔglnP1 strain, however, the CAT cassette is orientated in the opposite direction to the glutamine permease operon, resulting in the interruption of both promoters.


promoter region of glnP1
possible promoter element of glnP2
17874
csg
glnP1
glnP2
glnQ
Ω
KanR
Ω
P2?
Δcsg
csg
glnP1
glnP2
glnQ
CAT
glnP1
P1?
ΔglnP1
csg
glnP1
glnP2
glnQ
P2?

promoter element exists for the *glnP2* gene, then the operon would be able to be transcribed even if *csg* is also being transcribed (Fig. 4.3). This would undermine theories regarding the quick switching between metabolic processes during carbon starvation.

4.4 Analysis of the expression of *H. pylori csg*

4.4.1 Analysis of the expression of *H. pylori csg* by studying RNA

Analysis of *csg* RNA to study *csg* expression proved to be unsuccessful for two possible reasons. The first possibility is that RNA was degraded during the northern blotting process which failed to detect *csg* mRNA, and even the positive control. As this was a realistic possibility, all RNA solutions were replaced to ensure that no RNAse was present that could lead to the degradation of RNA. Despite replacing these solutions, no results were obtained from northern blots.

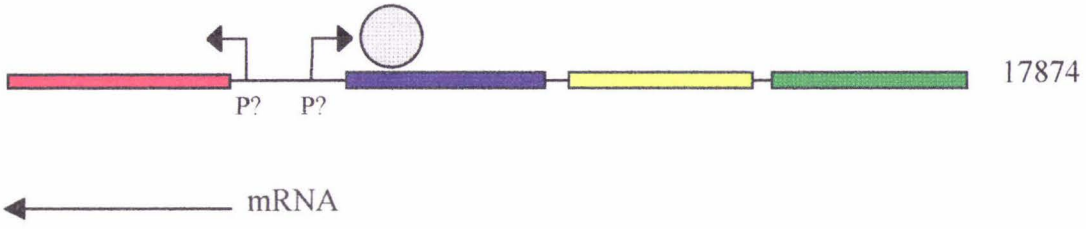
The second possibility for the failure of analysing *csg* RNA was that not enough RNA was isolated from each sample due to the small sample volumes. This same problem may have occurred when isolating RNA for RT-PCR to study transcript of the glutamine permease operon (Section 3.3.5).

As a more sensitive, reliable system was required to enable the study of *csg* expression, it was decided to construct a plasmid with a reporter gene placed under the control of the *csg*

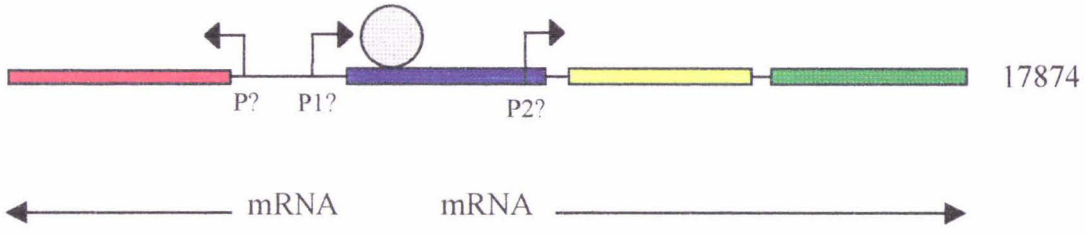
Figure 4.3 Implications for the theory of mutually exclusive *csg* and glutamine permease operon transcription

If the theory that *glnP2* possesses a promoter element is correct, there are implications for the theory of mutually exclusive *csg* and glutamine permease operon transcription. This figure shows the two possibilities. Figure a. shows what was previously hypothesised, that the *glnP1* promoter was responsible for the transcription of *gln2P* and *glnQ*. When cAMP binds to the putative CRP binding site in the *glnP1* sequence, it was thought that only *csg* could be transcribed. However, if a promoter element exists for *glnP2*, as seen in figure b., then the binding of cAMP will inhibit the transcription of *glnP1*, but not that of *glnP2/Q*. As a result, the glutamine permease transporter would not be affected.

a.



b.



promoter. The promoterless CAT gene was chosen for this, as a sensitive CAT ELISA assay is available commercially (Boehringer Mannheim).

4.4.2 Problems in the construction of a CAT expression vector

A variety of problems were encountered in the construction of the CAT expression vector for *csg*. The most common problem encountered was the low yields of DNA from gel purifications. DNA was lost at every step of the initial strategy, as the purified antibiotic resistance cassettes were ligated and the band of the desired size was gel purified. All ligations of this purified fragment with the pAB04 vector DNA proved unsuccessful.

To combat the problem of DNA loss, ligations were performed in the gel slice to minimise the loss of DNA due to gel purification. This, too, proved unsuccessful, most likely due to the large volumes of melted gel slice required for optimum vector:insert ratios. As a result, the gel was not diluted sufficiently to remain liquid. Three way ligations were also attempted to avoid losing DNA while attempting to ligate the CAT and Kan^R cassettes. However, gel purification of the CAT cassette resulted in low yields of DNA, which made this method initially unsuccessful.

After numerous attempts to excise and gel purify the CAT cassette, resulting in low DNA concentrations, a gel purification finally yielded good amounts of DNA (approximately 30ng/µl). A subsequent three way ligation reaction resulted in five plasmids containing the CAT and Kan^R cassettes, two of which contained the cassettes in the desired orientation.

In all the attempts to construct this plasmid, it appears that the concentration of the gel purified CAT cassette was the limiting factor. It is not known why gel purification of this fragment containing the CAT cassette yielded such low concentrations of DNA in comparison with the gel purification of other fragments. This may have been due to the small size of the fragment, such that gel purification resulted in a similar molar concentration as that of the larger fragments.

The new plasmid was transformed into *H. pylori* 17874 and a representative transformant was put into glycerol stocks. Due to time constraints, any further experiments to investigate the expression of *csg* were not possible. It is hoped, however, that experiments will be carried out to investigate the expression of *csg* under various environmental conditions. As there appears to be little change in phenotype during carbon starvation, it would be of interest to discover at what stage in cell growth that *csg* is expressed.

5.0 Summary and conclusions

The *csg* and *glnP1* genes of *H. pylori* 17874 were sequenced in both strands. Analysis of the sequence data showed the *csg* gene to have a putative σ^{70} promoter, as did the *glnP1* gene. The putative CRP binding site for the possible regulator of *csg* is located in the 5' sequence of *glnP1*, suggesting that the two genes are co-regulated. PCR analysis of the two genes has found them to be linked in eleven out of eleven *H. pylori* strains, but not in a *H. mustelae* strain.

A construct was made that lacked the promoter region of both *csg* and *glnP1*, as well as the first 50bp of sequence of each gene. The promoter regions were replaced with *Bgl*II recognition sequence. The KanΩ cassette was cloned into the new *Bgl*II site and the new plasmid was transformed into *H. pylori* 17874. Phenotypic analysis was carried out on this new strain to determine the effect of a non-functional *csg* gene. As the *glnP1* gene was also non-functional, phenotypic analysis was also performed on an existing strain that contained a non-functional *glnP1* gene. The ΔglnP1 strain was not able to grow in defined medium, but the Δcsg strain was, despite possessing non-functional a *glnP1* gene. This suggested that *glnP2* may possess a promoter element in the 3' region of *glnP1*, which would enable transcription of *glnP2/Q* in Δcsg but not in ΔglnP1, as the insertion of the CAT cassette disrupts transcription. However, no experimental evidence was obtained to support this theory.

The Δcsg strain was found to have a slower growth rate than the wild type in defined medium, suggesting that the role of Csg was to scavenge for substrates to increase the carbon sources available to the cell during carbon starvation. This agrees with the conclusions drawn by Schultz and Matin (1991) regarding the *cstA* gene of *E. coli*, the homologue of *csg*. It can also be concluded that the *csg* gene is not vital to cell growth *in vitro*, but does allow quicker growth in media with low carbon concentrations. As such, this gene may be important *in vivo* where carbon sources are often limited and quick growth may prove vital to colonisation.

A *csg* mutant was made that will allow the study of colonisation of *H. pylori* with a non-functional *csg* gene in the mice. pAB03 was transformed into *H. pylori* Sydney strain. Colonisation studies will be carried out by Dr Wangxue Chen of the Wakefield Gastroenterology Research Institute, Wellington, NZ.

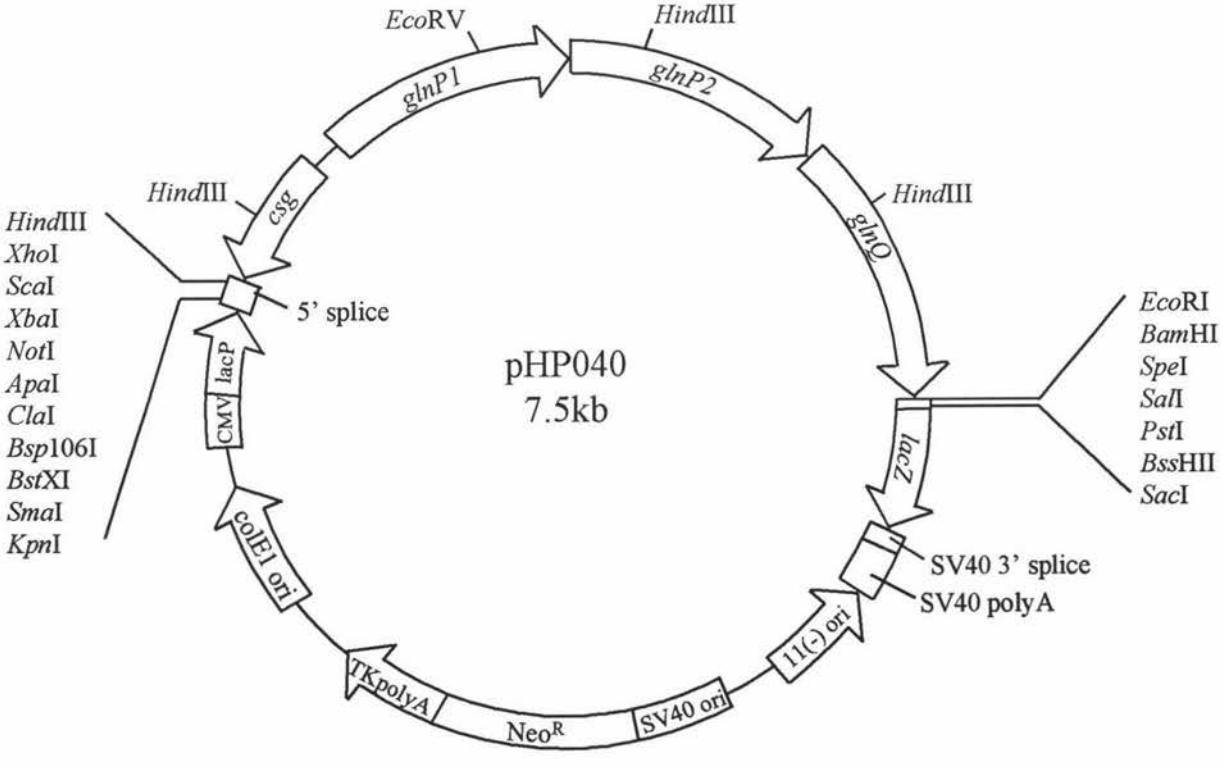
A plasmid was constructed with a promoterless reporter gene (CAT) placed under the control of the *csg* promoter. The CAT cassette was cloned into the *Bgl*II site of pAB04. To select for the presence of the CAT cassette, Kan^R was also cloned into pAB04 in a three way ligation. This plasmid was transformed into *H. pylori* 17874 and transformants were selected on the ability to grow on kanamycin. A representative transformant was stored in glycerol stocks and will be studied in this laboratory at a later date. This strain will aid in future study of the regulation of *csg* in various environments, such as an increase in the level of cAMP and a change in pH.

Appendices

Appendix A - Plasmid maps

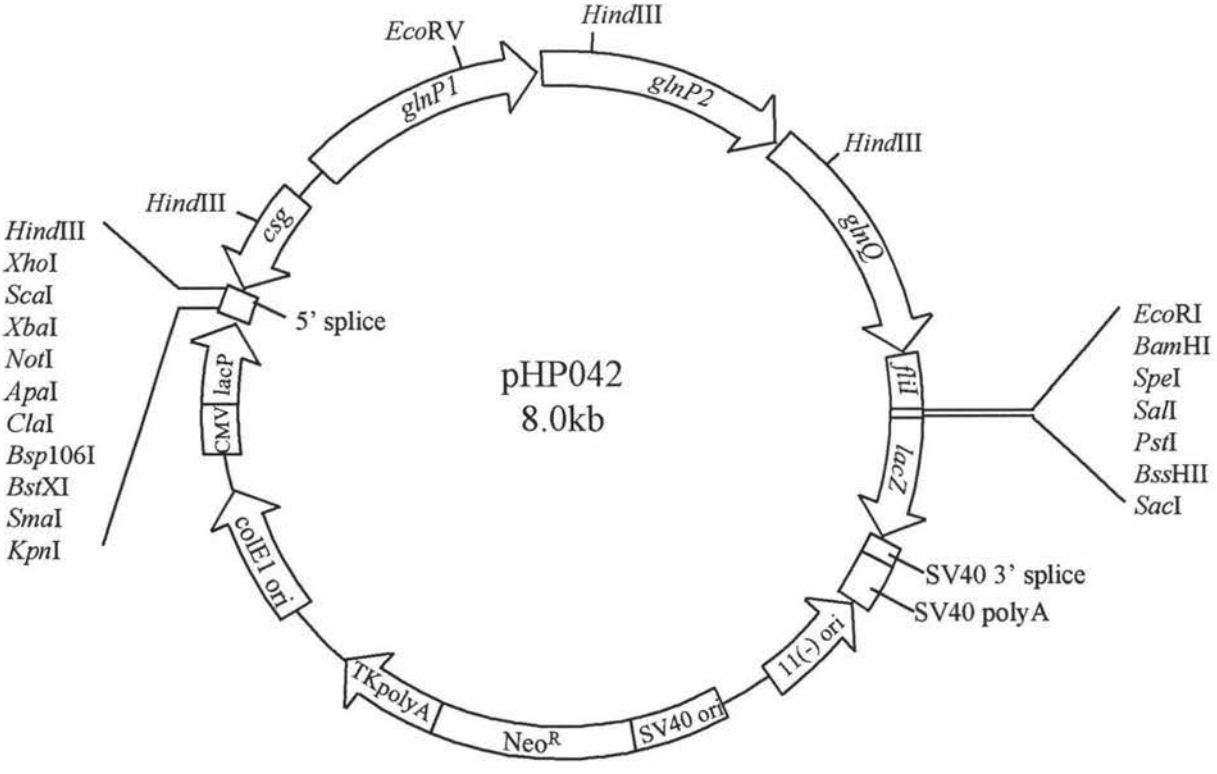
Appendix A.1

pHP040, constructed by Dr Paul O'Toole, contains a genomic insert of *H. pylori* in the pBK-CMV vector. The genomic fragment contains genes *csg*, *glnP1*, *glnP2*, and *glnQ*.



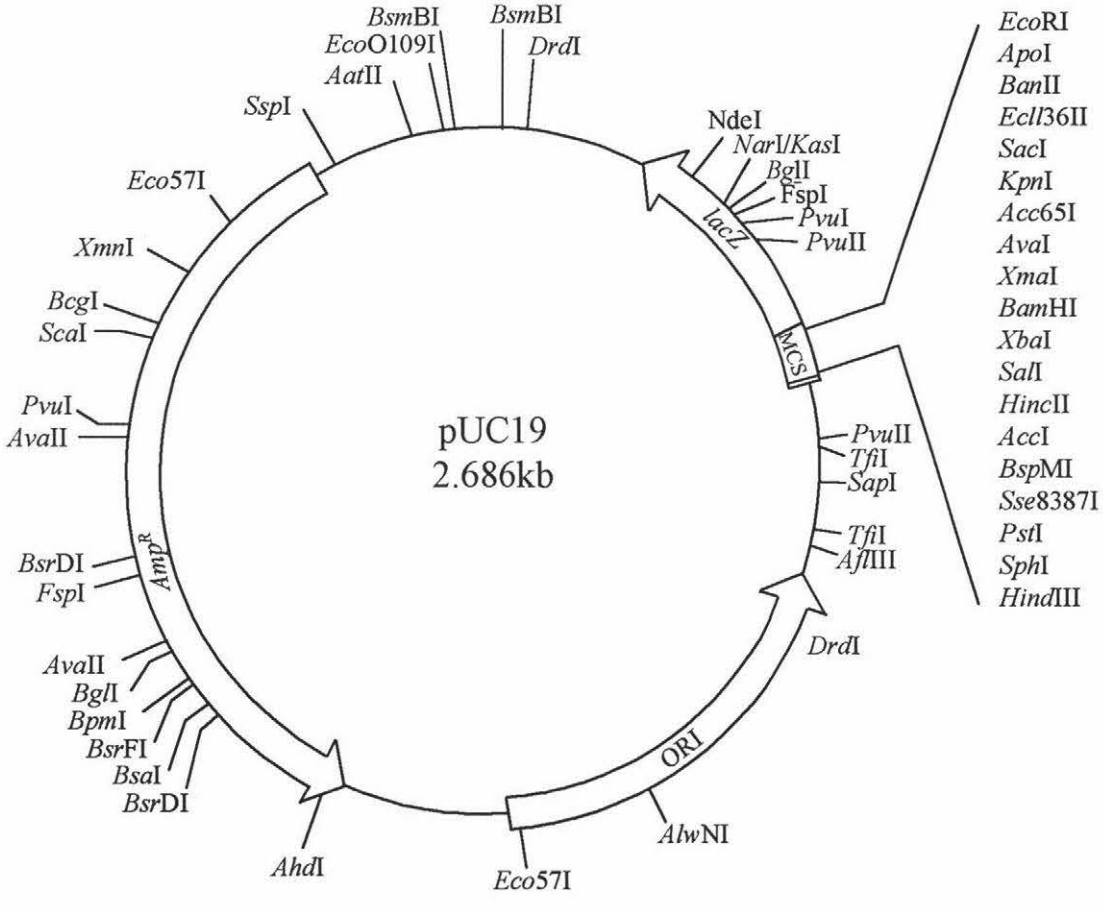
Appendix A.2

pHP042, constructed by Dr Paul O'Toole, is similar to pHP040 with two major differences. The *csg* gene is extended by approximately 400bp, and the *glnQ* is truncated. A fragment of *fliI* has also been cloned into pHP042, downstream of *glnQ*.



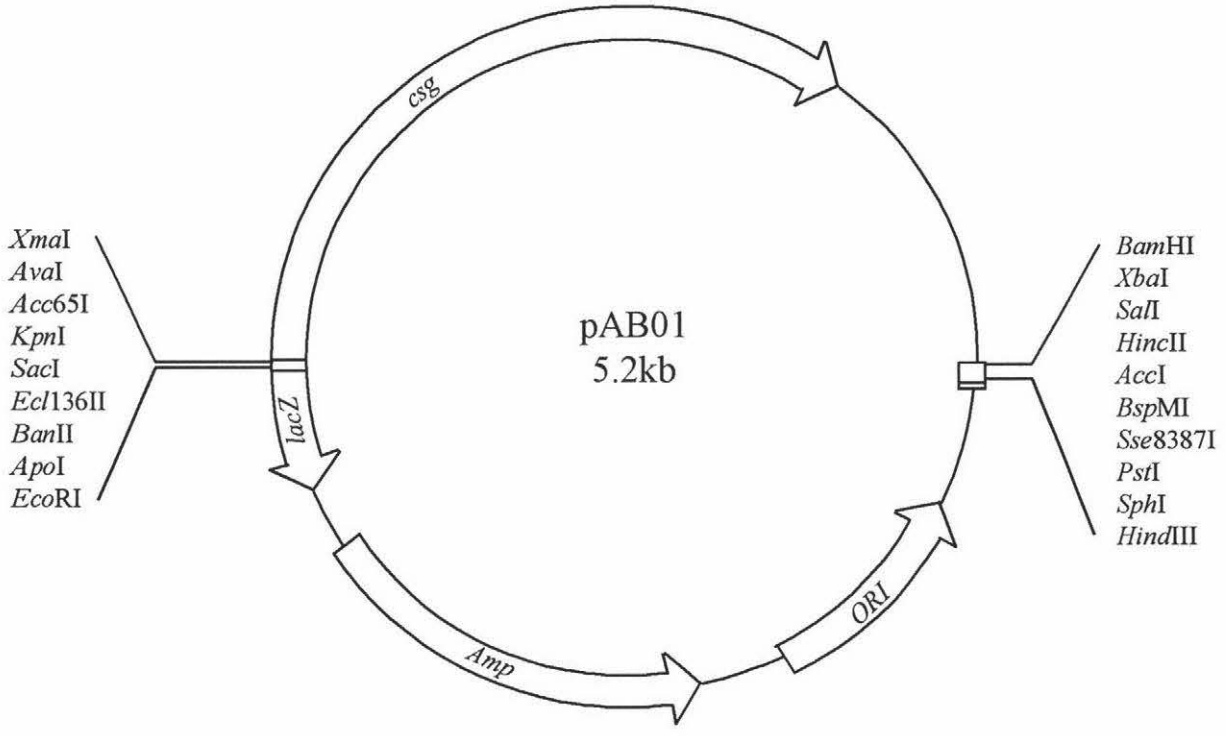
Appendix A.3

The plasmid map of pUC19, showing all restriction sites.



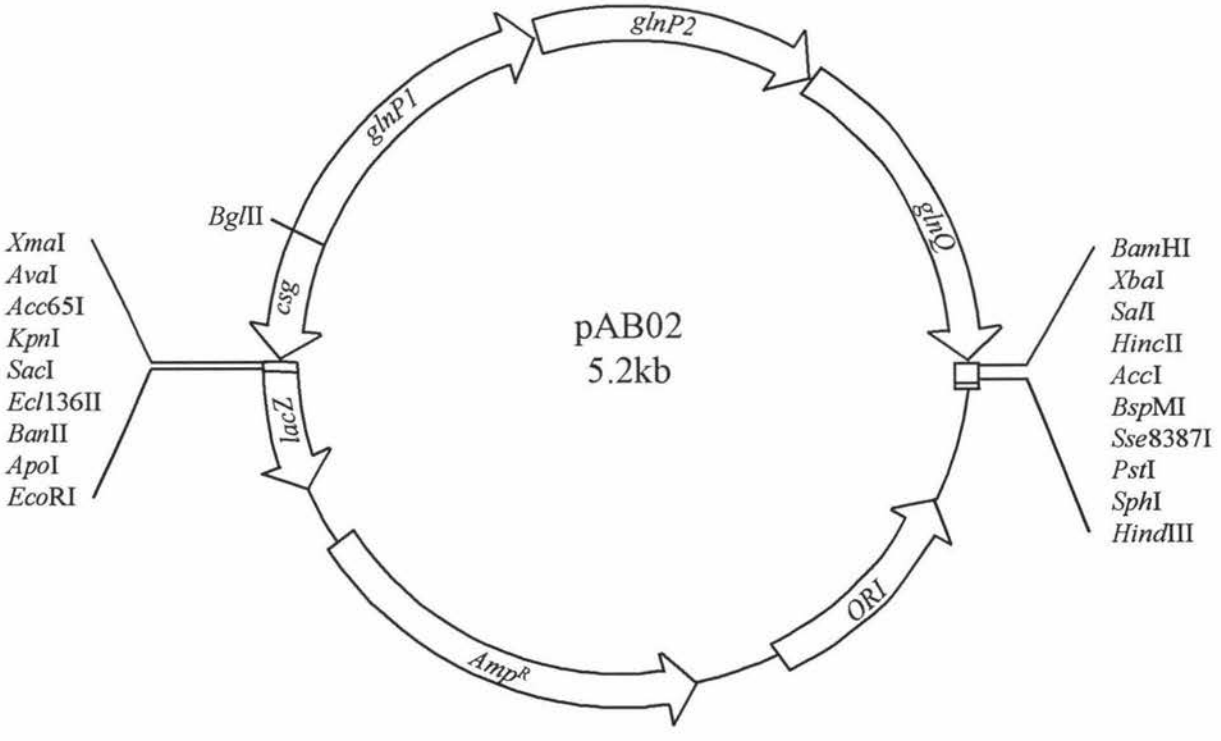
Appendix A.4

pAB01 was constructed to complete the sequencing of *csg*. The plasmid contains a genomic fragment from *H. pylori* 17874, which carries the 3' end of *csg*. The fragment was cloned into the *Sma*I site of pUC19.



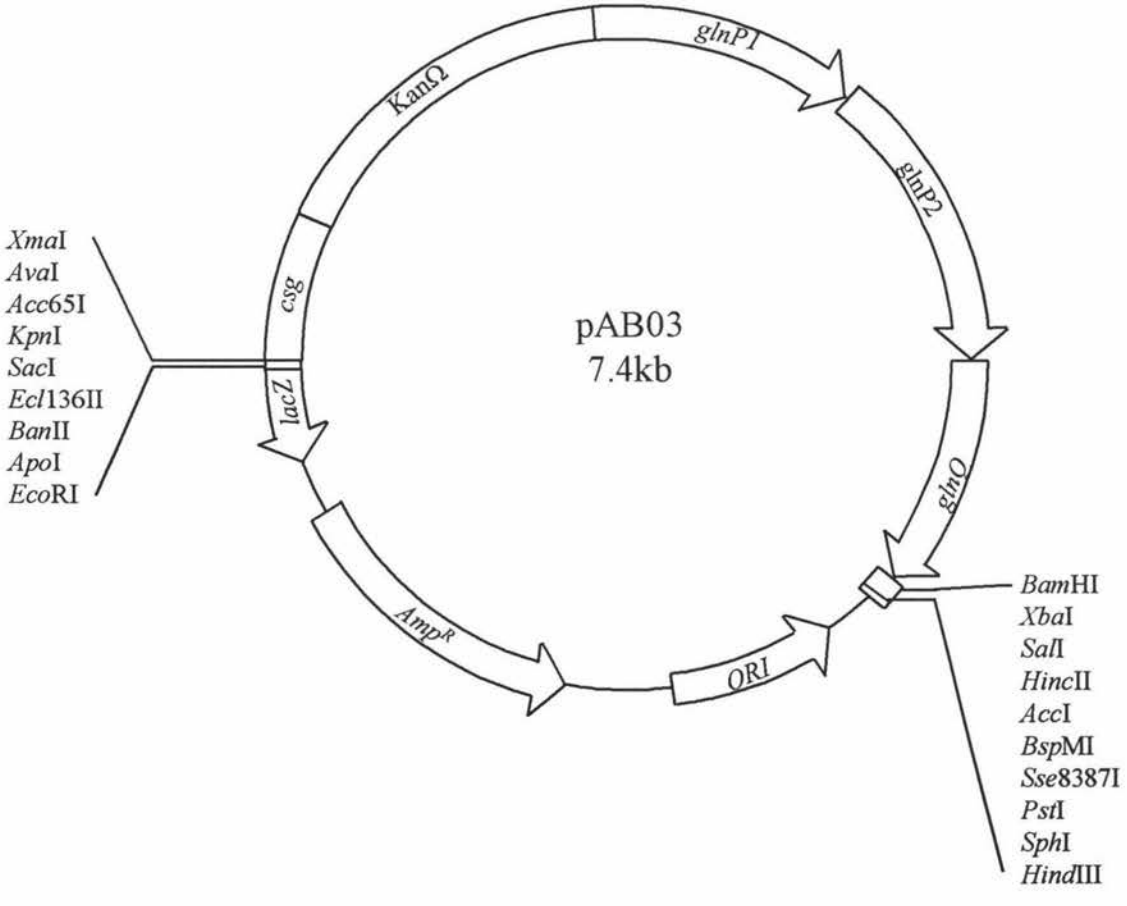
Appendix A.5

pAB02 was constructed as an intermediate plasmid during the mutagenesis of *csg*. The promoter regions of *csg* and *glnP1* was and replaced with a *Bgl*II recognition sequence. The fragment was cloned into the *Sma*I site of pUC19.



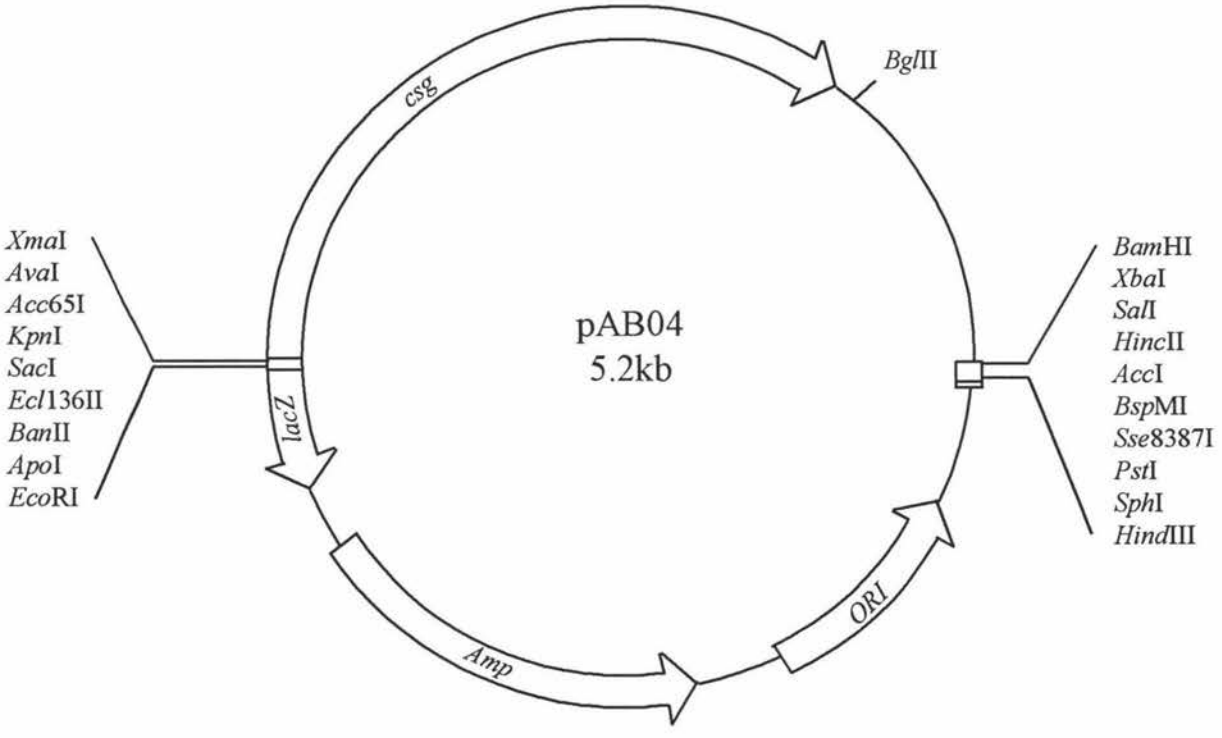
Appendix A.6

pAB03 is similar to pAB02 with the inclusion of the KanΩ cassette cloned into the *Bgl*II site of pAB02. The plasmid confers both ampicillin and kanamycin resistance.



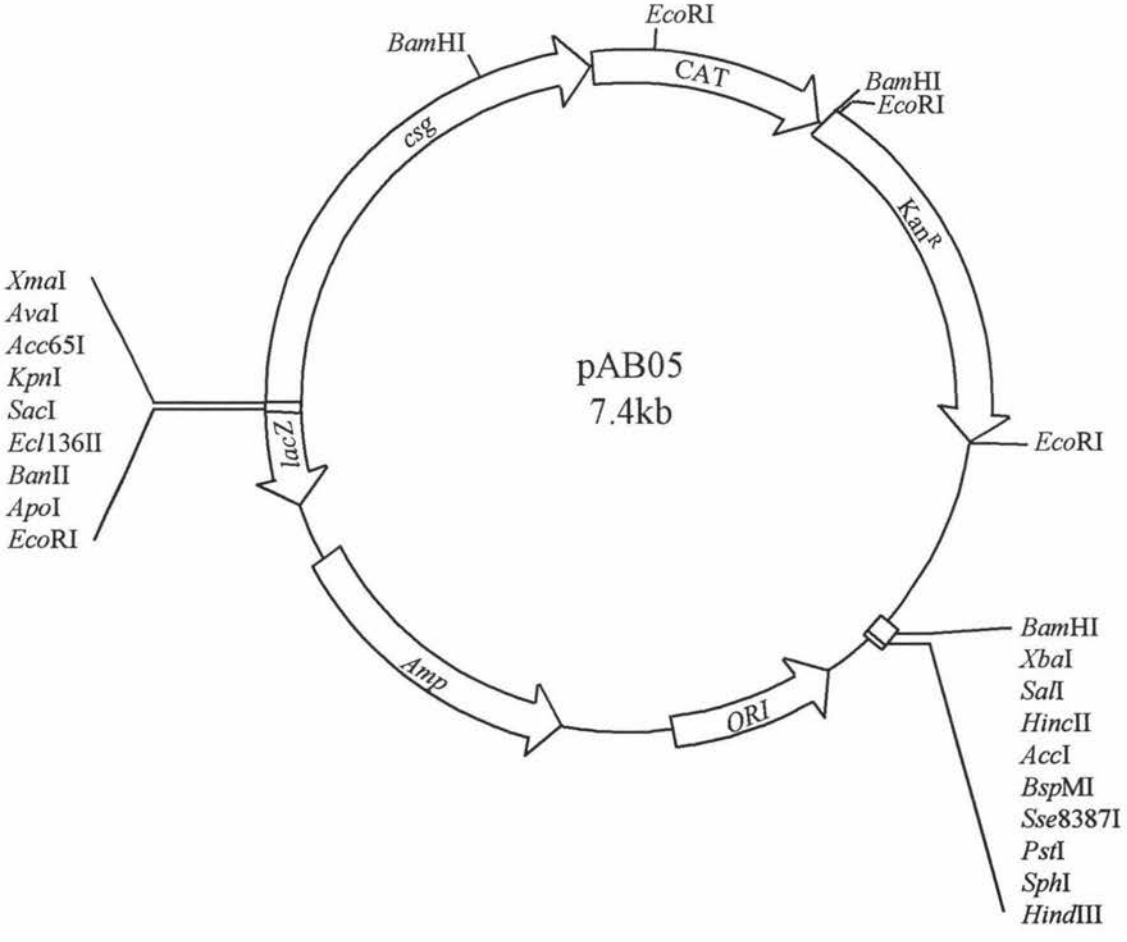
Appendix A.7

pAB04 is based on pAB01, with an engineered *Bgl*II site 10bp downstream of *csg* to allow a reporter gene to be placed under the control of the *H. pylori csg* promoter.



Appendix A.8

pAB05 contains a promoterless CAT cassette and a kanamycin resistance marker cloned into the *Bgl*II site of pAB04. The plasmid confers ampicillin and kanamycin resistance, but not chloramphenicol resistance.



Appendix B - Sequence data

Appendix B.1

The complete coding sequence of *H. pylori* 17874 *glnP1*. The sequence of the sense strand was available at the onset of this thesis work, however, the anti-sense strand was sequenced during this study and submitted to GenBank (Accession #U81796).

```
  1 ATGATTAAAC AATCATTAAA TGGAGAGGAC ATGCAAAAAA GTTTAGTTTC
 51 TTTGGCTTGG GTTTTTGTCG CTATTTTAGG GGCGATCTGT TAGGGGTGT
101 TAGCCTTACA CAAGGGTGAG AGCATCAACA CGCTATGGCT TGTAGTAGCG
151 AGCGCTTGCA TTTATAGCAT AGGCTATCGT TTTTATAGCC ATTTTATCGC
201 TTATCGGGTG TTAAAGCTAG ATGATAACAG AGCCACGCCT GCATGCGTAA
251 GGAATGACGG CAAGGATTTT GTGCCAACCG ATAAAGCCAT CACCTTTGGG
301 CATCATTTCG CCGCTATTGC TGGGGCTGGC CCTTTAGTAG GCCCGATACT
351 AGCCGCTCAA ATGGGTTACT TGCCCTCTAT CTTATGGATT TTGATAGGTT
401 CGGTTTTAGG GGGTTGCGTG CATGATTTTG TGGTGCTTTT TGCTTCTATT
451 AGGCGCGATG GCAAGTCTTT AGGCGAAATG ATCAAGCTTG AAATGGGTCA
501 ATTTGTAGGC ATGATCGCTA GTTTAGGGAT TTTAGGGATC ATGCTCATTA
551 TCATTGCGAT TTTAGCGATG GTGGTGGTGA AGGCTTTAGC GCATTCGCCT
601 TGGGGCTTTT TTACGATCGC TATGACCATT CCCATTGCGA TTCTTATGGG
651 GCTTTAC
```

Appendix B.2

The complete coding sequence of *H. pylori* 17874 *csg*.

```
   1 ATGCAAAAAA GTTTAGTTTC TTTGGCTTGG GTTTTTGTCG CTATTTTAGG
  51 GGCGATCTGT TTAGGGGTGT TAGCCTTACA CAAGGGTGAG AGCATCAACA
 101 CGCTATGGCT TGTAGTAGCG AGCGCTTGCA TTTATAGCAT AGGCTATCGT
 151 TTTTATAGCC ATTTTATCGC TTATCGGGTG TTAAAGCTAG ATGATAACAG
 201 AGCCACGCCT GCATGCGTAA GGAATGACGG CAAGGATTTT GTGCCAACCG
 251 ATAAAGCCAT CACCTTTGGG CATCATTTCG CCGCTATTGC TGGGGCTGGC
 301 CCTTTAGTAG GCCCGATACT AGCCGCTCAA ATGGGTTACT TGCCCTCTAT
 351 CTTATGGATT TTGATAGGTT CGGTTTTAGG GGGTTGCGTG CATGATTTTG
 401 TGGTGCTTTT TGCTTCTATT AGGCGCGATG GCAAGTCTTT AGGCGAAATG
 451 ATCAAGCTTG AAATGGGTCA ATTTGTAGGC ATGATCGCTA GTTTAGGGAT
 501 TTTAGGGATC ATGCTCATTA TCATTGCGAT TTTAGCGATG GTGGTGGTGA
 551 AGGCTTTAGC GCATTCGCCT TGGGGCTTTT TTACGATCGC TATGACCATT
 601 CCCATTGCGA TTCTTATGGG GCTTTACATG CGATTTTTCA GGCCGCATAA
 651 GATTTTGGAA GTTTCTGTTA TTGGCTTTAT CCTATTGATT ATAGCGATTT
 701 ATGCGGGTAA ATACATTTCT TTAGATCCTA AATTAGCGTC GATATTCACC
 751 TTTGATGCCA GTTCTTTAGC GTGGATGATC ATGGGTTATG GTTTTGTGGC
 801 TTCTATTTTG CCGGTATGGT TTTTACTCGC TCCACGAGAT TATTTAAGCA
 851 CTTTTTTAAA AATTGGCGTT ATAGGGGTGT TGGTTGTGGC TATTGTTTTT
 901 GTCGCTCCGC CCTTGCAAAT CCCTAAAATC ACGCCCTTTG TAGATGGCAG
 951 TGGGCCTGTA TTTGCAGGAA GCGTGTTCCC TTTCTTGTTT ATCACGGTGG
1001 CTTGCGGGAC GATTAGCGGC TTTCATGCTT TAATTTCTTC AGGAACGACC
1051 CCTAAAATGC TCGCTAAAGA AAGCGACGCA AGGCTAGTGG GCTATGGCTC
1101 TATGGTGATG GAGAGCGTTG TGGCTCTTAT GGCGTTGGTG TGCGCAGGGA
```

1151 TCTTGCACCC AGGGCTTTAT TTCGCTATCA ATTCGCCAGA AGTGAGCATC

1201 GGTAAAGATA TAGCTGATGC GGCTTCAGTG ATTAGCTCAT GGGGGTTTAG

1251 TATCAGCGCT GAAGAAATCA GTGAGATGAC TAAAAACATC GGCGAAAGCT

1301 CCATTTTGAG CCGCACCGGT GGGGCACCCA CTTTTGCGAT CGGTTTAGCG

1351 ATGATTGTGT ATCACATTTT AGGGGATCCA AGCGTGATGG CGTTTTGGTA

1401 TCATTTTGCG ATCTTGTTTG AAGCTTTGTT CATTTTAACC GCTGTGGATG

1451 CTGGCACACG AACCGCTCGT TTCATGATCC AAGATTTGCT CGGTAATGTT

1501 TATAAGCCTT TGGGTAATCT TAGCTCTTAT AAGGCTGGGA TTTTTGCCAC

1551 TCTTTTGTGC GTGGCAGGGT GGGGGTATTT CTTGTATCAA GGCACGATTG

1601 ATCCTAAAGG GGGGATTTAT ACGCTATGGC CTTTATTTGG CGTGAGCAAC

1651 CAGATGTTAG CGGGCATGGC GTTGTTGTTG GTTACGGTGG TGTTGTTTAA

1701 AATGGGGCGT TTTAAGGGGG CGATGATAAG CGCCTTACCG GCAGTTTTGA

1751 TTTTGAGCAT CACTTTTTAT AGCGGTATTT TAAAAGTGAT GCCAAAGAGC

1801 GATGATAGCG TGCTTAATAA TGTCTCGCAT GTGGCGCAAA TGCAAATCAT

1851 CAAAGAAAAA ATGGCTATTA CTACCGATGA AAAAGCGCTA AAAACGCTCC

1901 AAAAATCCTT TTTTAACCAT GCGATTGATG CGATTTTGTG CGTGTTTTTC

1951 ATGCTGGTAG CGCTTTTAGT GTTGATCGTG AGCGTTAGGA TTTGCTCAAA

2001 CGCTTATTTT AAAAACAAAA TTTACCCACC GCTGGCTGAA ACGCCCTATA

2051 TCAAAGCCAC TTGA

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