Identification of an immunogenic 18 kDa protein of *Helicobacter pylori* using alkaline phosphatase gene fusions

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Abstract

Secreted or surface-associated proteins play an important role in the immunopathogenesis of *Helicobacter pylori* infection. The aim of this study was to identify, using a genetic approach, *H. pylori* exported proteins and assess their role in the host immune response to infection. As part of this work, an *H. pylori* expression library was constructed and screened with a monoclonal antibody raised to a component of outer membrane vesicles from *H. pylori*, identified and characterised in a separate study. The screening strategy identified a locus of the genome containing two genes encoding exported proteins. Subsequent expression studies identified the gene product detected by the antibody as Lpp20, which encodes a well characterised lipoprotein from *H. pylori*. In addition, the use of alkaline phosphatase (AP) gene fusion methodology enabled the identification of a large number of other *H. pylori* exported proteins. Immunoscreening of a selection of enzymatically active *H. pylori* AP fusion proteins was carried out by Western blot analysis with patient sera and lymphocyte proliferation assays using peripheral blood mononuclear cells from *H. pylori* infected individuals. These assays identified a novel *H. pylori* exported antigen which was recognised by antibody derived from *H. pylori* infected individuals. Southern blot analysis revealed that the gene encoding the protein was absent in other *Helicobacter* species tested and sequence analysis of the gene demonstrated that it is highly conserved among *H. pylori* isolates. In order to obtain pure recombinant protein, the gene encoding the protein was cloned and expressed as a Beta-galactosidase (β-gal) fusion in *Escherichia coli* and the protein purified by affinity chromatography. The size of the recombinant protein released (18 kDa) was consistent with the calculated molecular mass of the polypeptide deduced from the DNA sequence. In Western blot assays, the purified protein was recognised by 71% of sera taken from patients infected with *H. pylori*, but by only 16% of sera taken from patients with unrelated or with no gastrointestinal disease. These results indicated that the 18 kDa protein from *H. pylori* was immunogenic and expressed *in vivo*. In other experiments, it was found that oral administration of this antigen did not protect mice against *H. pylori* colonisation following challenge with *H. pylori*.

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Chapter 1 Introduction

1.1 Gastrointestinal disease

1.1.1 Historical background

Ancient and medieval medical records describe the complications of peptic ulcer disease, but the first description of gastric ulceration was not reported until 1586 by an Italian physician (Donati, 1586; cited in Buckley and O'Morain, 1998). The symptoms of peptic ulceration were later described in 1825 and, with the ability to diagnose gastrointestinal disease, gastric surgery developed in the late nineteenth century. By the start of the twentieth century, the role of gastric acid in the development of ulcer disease was well established (Kidd and Modlin, 1998). The concept that acid imbalance was the primary cause of altered gastric pathology prevailed in most of the early twentieth century. As such, the treatment of peptic ulceration was based almost exclusively on the notion of neutralising or inhibiting the secretion of gastric acid, mostly through therapy with histamine H_2 receptor antagonists and other compounds such as prostaglandins, various antacid formulations and proton pump inhibitors. In addition, sucralfate, colloidal bismuth and carbenoxolone sodium were used to enhance local mucosal resistance without affecting gastric acid. However, in the face of mounting evidence that these treatment regimes were at best palliative, many investigators realised that peptic ulcer disease was a multifactorial process whose etiology was poorly understood.

The presence of gastric spiral bacteria in the canine stomach was first reported in 1893, and numerous reports describing similar "spirochetes" in the stomach of humans are scattered throughout the following century (reviewed in Kidd and Modlin, 1998). As a result, a bacterial origin underlying gastric ulceration had been proposed, but demonstration of this was hindered by the problems associated with the isolation and culture of pure strains from the stomach and researchers were unable to culture any candidate organisms. As such, interest in these spiral bacteria diminished and the dictum "no acid, no ulcer" (Shwartz, 1910; cited in McNulty, 1999) remained. The initial report implicating a Campylobacter-like organism in the aetiology of human gastritis and peptic ulceration (Marshall and Warren, 1984) was thus met with much

scepticism. Their discovery, however, soon demanded the attention of researchers working in the field of gastroenterology.

1.1.2 The discovery of *Helicobacter pylori*

The observation that spiral bacteria were commonly found during microscopic evaluation of biopsy samples taken from patients with gastritis initiated a study which would eventually redefine the treatment of peptic ulcers. The investigation of over one hundred and thirty gastric biopsy samples from gastritis patients confirmed the presence of this *Campylobacter*-like organism and the consistency of the associated histological changes, fulfilling Koch's first postulate (Goodwin *et al.*, 1986). However, using *Campylobacter* isolation techniques, attempts to culture this bacteria under microaerophilic conditions with a limited incubation of two days were unsuccessful. Serendipity intervened, and the thirty-fifth culture was left to incubate for an extended period over the Easter weekend, resulting in the growth of a pure bacterial colonies (Marshall and Warren, 1984).

In order to demonstrate that this gastric bacterium was the etiological agent of human gastritis, Marshall *et al.* (1985) and Morris and Nicholson (1987) independently satisfied Koch's postulates in two separate experimental human ingestions of the bacteria. Following ingestion of 10^9 colony forming units (CFU), acute gastritis, characterised by polymorphonuclear cell infiltration, mucus depletion and epithelial cell damage, was demonstrated histologically (Marshall *et al.*, 1985). This newly described bacterium was originally named *Campylobacter pyloridis*, and later *Campylobacter pylori*, as a result of initial morphological similarities to the *Campylobacter* bacteria. However, this was changed to *Helicobacter* in 1989 when the bacteria were recognised as a separate genus with the discovery of their unique fatty acid profile and later by ribosomal RNA sequencing (Romaniuk *et al.*, 1987). The discovery, and subsequent studies on this human pathogen, known currently as *Helicobacter pylori*, revolutionised the treatment and diagnosis of gastrointestinal disease.

1.2 *Helicobacter* species

1.2.1 Classification

The *Helicobacter* group is classified in the Proteobacteria, a large, diverse division of gram-negative bacteria. In a phylogenetic tree derived by maximum likelihood analysis of 16s subunit rRNA sequences from 253 representative species of bacteria (Olsen *et al.*, 1994), *H. pylori* was placed in the Epsilon subdivision, which also includes the *Campylobacter* group. *Helicobacter pylori* was the first characterised member of this genus. Morphologically, *Helicobacter* species range from the gently spiralled *H. pylori,* to the straight rods of *H. mustelae.* When grown on solid medium, *H. pylori* assume a more rod-like shape and spiral shapes are infrequently observed (Marshall *et al.*, 1985). After prolonged culture on solid or in liquid media, coccoid form tend to predominate (Benaissa *et al.*, 1996). Coccoid forms are metabolically active, however they cannot be cultured *in vitro* (Dunn *et al.*, 1997) and may represent a temporary dormant state. Both spiral and coccoid forms have been shown to cause acute gastritis in mice (Wang *et al.*, 1997). Others have demonstrated that adverse changes in DNA, RNA, membrane potential and protein synthesis in coccoid forms are consistent with bacterial cell death (Kusters *et al.*, 1997). Recent data suggests that the antigenic profiles between the two forms differ and may represent an immune evasion tactic (Benaissa *et al.*, 1996) by the bacterium. The significance of these coccoid forms is still under debate.

The main morphological feature of the *Helicobacter* genus is the possession of sheathed flagella, which are essential for motility (Haas *et al.*, 1993)(see also section 1.4.1). *H. pylori* has four to six unipolar sheathed flagella, each approximately 30 µm long and 2.5 nm in width (Geis *et al.*, 1989). The cell wall membrane is smooth in comparison to the rugose membrane of *Campylobacter* species. Most of the *Helicobacter* species also possess a potent urease activity which provides a primary biochemical distinction from other oxidase- and catalase-positive spiral organisms (reviewed in Dunn *et al.*, 1997).

1.2.2 The *H. pylori* genome

The entire *H. pylori* genome sequence was first published in September 1997, and described the genetic characteristics of *H. pylori* strain 26695, an isolate from a gastritis patient from the United Kingdom, chosen for its toxigenicity and ability to elicit an

immune and inflammatory response in piglets (Tomb *et al.*, 1997). Later, in February 1999, a second *H. pylori* genome was published describing *H. pylori* strain J99, isolated from a patient with duodenal ulcer from the USA (Alm *et al.*, 1999). A comparison of these genome sequences revealed that, despite evidence of a large degree of genomic and allelic diversity (Taylor *et al.*, 1992; Bukanov and Berg, 1994; Jiang *et al.*, 1996), the overall genomic organisation, gene order and encoded proteins was very similar (Alm *et al.*, 1999).

The genome size of *H. pylori* ranges from 1.6 to 1.75 Mb, with an average G+C content of 39% (Tomb *et al.*, 1997). The genome is circular and encodes approximately 1,500 open reading frames (ORFs). Sequence analyses of the *H. pylori* genome have revealed that *H. pylori* has well-developed systems for motility, scavenging iron and for DNA restriction and modification (Tomb *et al.*, 1997) as well as a complex system of enzymes (reviewed in Nilius and Malfertheiner, 1996). *H. pylori* also appears to encode a large number of outer membrane proteins and several regions of the genome contain a lower percentage of G+C than the rest of the genome, and may represent pathogenicity islands (see section 1.4.2). Although providing new insights into the genetic diversity of *H. pylori* (Huynen *et al.*, 1998), the limitation of this raw data is highlighted by the fact that approximately one third of the predicted coding sequences are not assigned even putative identifications. In addition, for many of the sequence identifications, a biological role was assigned on the basis of database matches of less than 50% similarity (Owen, 1998). As a result, many elements of pathogenesis still remain unclear at the molecular level, and further investigation into both bacterial and host factors is required.

1.3 *Helicobacter pylori* infection in humans

1.3.1 Epidemiology

H. pylori is now recognised as one of the most common human bacterial infections, with infection rates of around 50% in developed countries and approaching >90% in some developing countries (reviewed in Bardhan, 1997). There is now much evidence to suggest that *H. pylori* has been part of the normal microbiota of humans and our ancestors for millions of years (reviewed in Blaser, 1998). The recent finding of *H.*

pylori antigens in stools from South American mummies about 1,700 years old (Correa *et al*., 1998) is consistent with this hypothesis. The genetic diversity of *H. pylori* (Go *et al*., 1996), as well as clonal characteristics of *H. pylori* related to their geographical origin (Campbell *et al*., 1997) is also consistent with the prolonged presence of *H. pylori* in population groups, and against their recent spread to humans. In New Zealand, serological studies have found infection rates ranging from 36 – 73% depending on ethnicity. A high seroprevalence of *H. pylori* exists in Maori and Pacific Island groups, as opposed to European ethnic groups. Ethnicity as a risk factor for infection in New Zealand appears to be independent of socio-economic status (Fraser *et al*., 1996).

In concert with other enteric pathogens, childhood represents the phase of maximum susceptibility to infection with *H. pylori* (Cullen *et al*., 1993). The large differences observed between the developing and developed world in the acquisition of *H. pylori* infection (Bardhan, 1997), suggest the possibility of an environmental pool of *H. pylori* to which children are exposed in developing countries. However, in contrast to other enteric bacteria, the presence of *H. pylori* in the environment has been difficult to demonstrate. *Salmonella*, *Shigella*, *Vibrio cholerae* and more recently, *Campylobacter jejuni* are easily recovered from the environment, including from vectors, sewerage, and foodstuffs. Several investigators have attempted to detect *H. pylori* in water supplies both directly and indirectly, but viable *H. pylori* have not yet been cultured from such sources. *H. pylori* DNA has been detected in water supplies using the polymerase chain reaction (PCR) (Hulten *et al*., 1996).

No predominant route of acquisition of *H. pylori* has been defined. Possibilities include oral-oral, fecal-oral and vector transmission (reviewed in Mitchell, 1999). Data in support of oral-oral transmission comes from a variety of studies in which the bacteria have been cultured from the mouth (Dunn *et al*., 1997) and detected in saliva and dental plaque (Riggio and Lennon, 1999) using the PCR. There is little data to support the transmission via the fecal-oral route, as detection of *H. pylori* in faeces has proven technically difficult, with the exception of one report on the isolation of *H. pylori* from the faeces of children (Thomas *et al*., 1992). The potential for flies as vectors for enteric disease has been known for decades, and recently the housefly was also shown to have the potential for mechanistic transmission of *H. pylori* (Grubel *et al*., 1997).

Non-human primates have been shown to be colonised with *H. pylori*-like organisms, however, the limited interaction between these animals and humans, makes this an unlikely reservoir for human *H. pylori*. Domestic animals carry gastric *Helicobacter*-like organisms (GHLOs), but most of these have been identified as *H. heilmannii* (Neiger *et al.*, 1998; El-Zaatari *et al.*, 1997). Nevertheless, the identification of *H. pylori* in a colony of domestic cats (Fox *et al.*, 1995) and reports of *H. pylori* isolated from domestic cats suffering from moderate to severe lymphofollicular gastritis (Handt *et al.*, 1995) may have public health implications (see also section 1.6.1).

1.3.2 Clinical manifestations

H. pylori infection in humans can lead to a variety of clinical consequences, including peptic ulceration, gastric adenocarcinoma and gastric lymphoma. Only a proportion of individuals will experience these clinically significant disease manifestations. All infected individuals will develop chronic gastritis, usually without any detectable adverse symptoms.

1.3.2.1 Gastritis and peptic ulcer disease

Primary infection with *H. pylori* produces an acute gastritis, which is generally asymptomatic, but may be associated with mild nonspecific upper gastrointestinal symptoms. One feature of the acute infection with *H. pylori* is the development of hypochlorhydria, which has been observed in volunteers experimentally infected with *H. pylori* (Marshall, 1985; Morris and Nicholson, 1987), and is accompanied by a marked decrease in acid secretion. Despite the persistence of the bacterium, this acute hypochlorhydria generally resolves within several months. This transient decrease in acid secretion is believed to allow survival of the organism early in the natural history of infection (McGowan *et al.*, 1996). The normal gastric mucosa contains only a few scattered mononuclear cells, and lacks lymphoid follicles and polymorphonuclear (PMN) cells (Howden, 1996). *H. pylori*-associated gastritis is characterised by an abundant inflammatory response (see section 1.7) and gastric epithelial cell injury. The gastric inflammation that results is characterised by a predominance of PMN cells infiltrating the gastric antral mucosa. Acute *H. pylori* infection progresses to a chronic gastritis, characterised by an infiltration of monocytes/macrophages, lymphocytes and

plasma cells into the lamina propria (Kuipers, 1997). This chronic gastritis often leads to atrophic gastritis (Sakagami *et al.*, 1996), which, over time, may progress to metaplasia, and a decline in the numbers of *H. pylori* infecting the mucosa (McGuigan, 1996).

Other common histopathological findings are a marked reduction or loss of the mucin layer and epithelial erosive lesions that range from the focal loss of integrity of a few cells to extensive desquamation of the epithelial cell layer and exposure of the basal membrane to the gastric environment (Ghiara *et al.*, 1995). *H. pylori* infection is associated with nearly all duodenal and gastric ulcers formed independently of the ingestion of nonsteroidal anti-inflammatory drugs (Blaser, 1992). The estimated life-time risk for peptic ulcer disease in *H. pylori* infected individuals is approximately 15% (Kuipers, 1997). An individual's risk of ulcer disease depends upon the severity of gastritis, which is determined by various host and bacteria-related factors.

Among bacterial factors, diversity among *H. pylori* strains is believed to contribute to the various outcomes of infection. Expression of a vacuolating cytotoxin is more common among patients with peptic ulcer disease than among *H. pylori*-infected patients with superficial gastritis alone (Cover *et al.*, 1990). Similarly, strains harbouring the cag pathogenicity island (cag PAI) are more commonly isolated from peptic ulcer patients (Censini *et al.*, 1996). Gastric epithelial damage induced by a vacuolating cyotoxin (VacA) (Telford *et al.*, 1994) may result in increased access of acid to host tissues or alter the function of gastric parietal cells. In addition, strains possessing the cag PAI are associated with increased epithelial cell IL-8 production and increased gastric inflammation (Crabtree *et al.*, 1994) leading to further tissue damage (see also sections 1.4.2 and 1.7.1).

Among host factors, most of the evidence focuses on acid production in response to infection. Increased acid secretion limits *H. pylori* gastritis to the antrum, at the risk of duodenal ulcer disease, whereas reduced acid secretion allows a more proximal inflammation at the risk of atrophic gastritis, gastric ulcer disease and gastric cancer (Kuipers, 1997). The response to infection is determined by host and geographical factors including genetic make-up, age at infection, nutritional status and diet (reviewed

in Leon-Barua *et al.*, 1997). Thus, the outcome of infection, in terms of disease, will differ both within and between populations, particularly where these are subject to widely differing environmental influences (Goldstone *et al.*, 1996). The gastric antrum plays an important role in the regulation of normal gastric secretion (reviewed in McGowan *et al.*, 1996). G cells in antral mucosa produce gastrin, which stimulates acid secretion and is a growth factor for parietal cells. D cells are also found in the antrum and secrete somatostatin which inhibits gastrin release and acid secretion. Individuals infected with *H. pylori* who develop duodenal ulceration have two disturbances of gastric function: an increased release of gastrin from the antral mucosa and an exaggerated acid response to stimulation by gastrin compared with infected individuals without ulceration (McGowan *et al.*, 1996). The exaggerated response to gastrin may be the result of increased gastrin release from the G cells, possibly in combination with a decrease in mucosal somatostatin produced by the D cells (Veldhuyzen van zanten and Lee, 1999). This imbalance leads to an increased duodenal acid load resulting in gastric metaplasia in the duodenum that can then be colonised by *H. pylori*, leading to duodenitis and eventually duodenal ulcer (Olbe *et al.*, 1996).

1.3.2.2 *H. pylori* associated neoplasia

Gastric cancer is the second leading cause of cancer worldwide (Forman, 1998) and is the most frequently diagnosed malignancy in Japan (Pisani *et al.*, 1997). An epidemiological association between *H. pylori* and gastric cancer has now been well established (reviewed in Goldstone *et al.*, 1996 and Queiroz *et al.*, 1999) with infection estimated to increase the risk of cancer approximately nine-fold (Forman, 1998). Several retrospective case-controlled studies have confirmed a significant relationship between the prevalence of *H. pylori* antibodies and gastric cancer rates (Forman *et al.*, 1991; Parsonnet *et al.*, 1991; Nomura *et al.*, 1991; The Eurogast Study Group, 1993). The natural history of the association between *H. pylori* and gastric cancer appears to involve the acquisition of *H. pylori* in childhood leading to long term infection characterised by chronic gastritis with eventual progression in some individuals to atrophic gastritis and gastric mucosal atrophy (Leon-Barua *et al.*, 1997). Gastric mucosal atrophy is widely considered to be a precursor lesion of the intestinal-type gastric cancer (Correa, 1992), although *H. pylori* has found to be associated with both intestinal and diffuse types of gastric cancer (Hansson *et al.*, 1995). Atrophic gastritis is

a multifactorial disease. It has been suggested that *H. pylori* infection, along with genetic and environmental factors, including diet and excessive salt intake probably combine to exert a long term influence which leads to the progression of gastric mucosal atrophy to intestinal metaplasia, dysplasia and finally gastric adenocarcinoma (reviewed in Genta and Rugge, 1999). In addition, chronic *H. pylori* infection has been shown to be responsible for genomic instability in gastric cells, which is generally associated with the phenotypic expression of precancerous lesions (Nardone *et al.*, 1999; Solcia *et al.*, 1996), increased apoptosis of epithelial cells (Rudi *et al.*, 1998) and increased gastric cell proliferation (Fan *et al.*, 1996). The interactions of proinflammatory factors and phagocytes leads to the production of a variety of reactive oxygen metabolites causing oxidative stress, which also has a proven link with carcinogenesis (Goldstone *et al.*, 1996). The accumulated evidence for a role of *H. pylori* infection in gastric cancer led the International Agency for Research on Cancer to identify *H. pylori* as a Class I carcinogen (IARC, 1994), making *H. pylori* the first bacterial infection recognised as a human carcinogen.

In addition to the strong statistical association with gastric adenocarcinoma, *H. pylori* infection has been associated with low-grade B cell gastric lymphoma of the mucosa-associated lymphoid tissue (MALT) (reviewed in Wotherspoon, 1998 and Bayerdorffer *et al.*, 1997) Primary gastric non-Hodgkin's lymphoma comprises 3-6% of all gastric malignant tumours, and are typically high-grade B cell lymphomas, but a significant proportion are low grade tumours (Wotherspoon, 1998). Several studies have shown that infection with *H. pylori* is associated with the accumulation of MALT and non-infected individuals rarely have such tissue in their gastric mucosa (Bayerdorffer *et al.*, 1995). This lymphoid formation seems to be a universal response to *H. pylori* irrespective of CagA or VacA status (Witherell *et al.*, 1997). A causal role for *H. pylori* infection in B cell MALT lymphoma has been well documented and *in vitro* studies have demonstrated that the proliferation of tumor cells in low-grade gastric MALT lymphoma is driven by the presence of the organism and is T cell dependent (Hussell *et al.*, 1993). Of particular importance, clinical studies have shown that, in early lesions, eradication of *H. pylori* can result in tumor regression in 60-92% of cases (Wotherspoon *et al.*, 1993; Wotherspoon, 1998). Relapse of the lymphoma has been reported in some cases, but this may be associated with recrudescence or reinfection by *H. pylori* (Stolte

et al., 1996).

1.3.2.3 Extradigestive diseases

Several studies have been carried out on the association between *H. pylori* and a miscellany of extradigestive diseases, such as cardiovascular disease (Ellis, 1997), immunological disorders (Gasbarrini *et al*., 1999) and various other conditions including skin diseases, liver and biliary tract disorders and low growth rate (Raymond *et al*., 1994). Hypochlorhydia resulting from *H. pylori* infection may also predispose an individual to enteric infections, particularly in underdeveloped countries (McColl, 1998). However, in many studies the data is conflicting and is complicated by the confounding effects of socioeconomic status. Well designed studies are required to clarify the existence of a causal association between *H. pylori* and other diseases. Recent studies have suggested that *H. pylori* infection may even protect individuals from gastroesophageal reflux disease and oesophageal adenocarcinoma (El-Serag and Sonnerberg, 1998). Corpus gastritis has been recently shown to be associated with a 54% reduction in the risk of reflux oesophagitis (El-Serag *et al*., 1999).

1.4 Pathogenesis of *H. pylori* infection

H. pylori occupies a unique niche in the mucus layer overlying the gastric epithelium and has adapted to the human stomach mucosa, where it can persist for years or even decades by apparently evading the host immune system. *H. pylori* has to survive amidst a strong humoral and cellular immune response and also an extremely hostile environment in the gastric mucosa. The organism possesses a variety of features (reviewed in Labigne and de Reuse, 1996) which enable it to survive in the stomach, and to colonise and persist long-term.

1.4.1 Colonisation and persistence in the gastric mucosa

One predominant feature of *H. pylori* is its ability to produce large amounts of the enzyme urease, which is an essential determinant of pathogenicity. Involvement of urease in the pathogenesis of *H. pylori* infection was first demonstrated by the inability of a urease-deficient mutant to colonise the gastric mucosa of the gnotobiotic piglet and mouse (Eaton *et al*., 1991; Tsuda *et al*., 1994). The urease protein of *H. pylori* is an

multimeric enzyme of 600 kDa, composed of two structural subunits (UreA) and (UreB), and is located in both the cytoplasm and on the surface of the bacteria (see section 1.9.2). In addition to the *ure*A and *ure*B genes, the entire urease operon of *H. pylori* contains seven accessory genes necessary for the assembly of a functional urease enzyme. The high urease activity of *H. pylori* is responsible for the hydrolysis of urea to ammonia and carbon dioxide. Although it is not clear yet how the enzyme acts, the ammonia produced by the organism may protect the bacteria and allow its survival in an acidic medium by buffering gastric acid or by increasing the local pH of the mucosal surface (Clyne *et al.*, 1995). It has also been demonstrated that the high levels of ammonia generated by urease activity have toxic effects on cells and may break down gastric mucous and alter its permeability (Smoot *et al.*, 1990).

All gastric *Helicobacter* species are highly motile organisms. *Helicobacter* species exhibit much higher motility in viscous media than any other enteric bacteria such as *Salmonella* or *E. coli.* Motility has been found to be essential for colonisation of intestinal surfaces by many gut pathogens (e.g. *Vibrio cholerae*, *Salmonella* sp. *Campylobacter jejuni*) and *H. pylori* is no exception. Studies using the gnotobiotic piglet as an experimental model have demonstrated that *H. pylori* relies strongly on its high motility to colonise and persist in the epithelium by showing that a nonmotile variant of a clinical isolate colonised poorly in contrast to the parental strain (Eaton *et al.*, 1996). Construction of isogenic flagellar mutants of *H. mustelae* also demonstrated the importance of flagellar motility as a virulence factor for colonisation in the ferret stomach (Andrutis *et al.*, 1997). The flagellae of *H. pylori* are unique in that they are encased in a membrane-like sheath. The sheath consists of a bilayered membrane that contains proteins, lipids and lipopolysaccharides and closely resembles the outer membrane, although differences, especially in fatty acid composition and protein patterns exist (Geis *et al.*, 1989). The function of the bacterial flagellar sheath is not well understood but it may have a role in flagellum-mediated adhesion to surfaces such as gastric epithelial cells.

H. pylori can interact with gastric epithelial cells by attaching to the apical membrane, producing an attaching-effacing effect similar to that observed in the small intestine with enteropathogenic *E. coli* (Smoot *et al.*, 1993). Expression of tissue-specific

adhesins is believed to be necessary for the colonisation of the human gastric epithelium by *H. pylori*. Adherence of *H. pylori* to the gastric mucosa is a fundamental stage of infection and several candidate receptors for *H. pylori* adhesin proteins have been described (reviewed in Logan, 1996). Like other bacteria which colonise the mucosal epithelium, *H. pylori* adheres to sialic-rich macromolecules that are exposed on the mucosal cell membrane and has a specific association with gastric-type epithelium. Several studies have attempted to characterise the haemagglutination (HA) of *H. pylori*, and have identified a 20 kDa surface protein with specificity for a sialic acid receptor (Evans *et al.*, 1995; Doig *et al.*, 1992) which was later suggested to be a 30 kDa flagellar lipoprotein (O'Toole *et al.*, 1995). A putative Lewis b binding adhesin of *H. pylori* has been identified (Ilver *et al.*, 1998) but its importance in mediating attachment to the gastric epithelium is still unclear. Once established in the gastric mucosa, *H. pylori* can then persist and damage the epithelium through a number of virulence determinants.

1.4.2 Virulence factors

The discrepancy between the prevalence of infection and occurrence of ulcers may be explained by the possible existence of ulcerogenic strains of *H. pylori*. To date, efforts to identify ulcerogenic bacteria have concentrated on those strains which possess the vacuolating cytotoxin (*vac*A) gene (Telford *et al.*, 1994; Phadnis *et al.*, 1994; Schmitt and Haas, 1994). Approximately 50% of clinical isolates produce active vacuolating cytotoxin (Cover *et al.*, 1990) and these isolates appear to correlate with the development of ulceration (Tee *et al.*, 1995; Zhang *et al.*, 1996). Several different families of *vac*A alleles have been identified (Atherton *et al.*, 1995), and infection of humans with strains containing the type s1m1 *vac*A alleles are associated with peptic ulceration (Atherton *et al.*, 1997; Rudi *et al.*, 1998; van Doorn *et al.*, 1998; Donati *et al.*, 1999) and gastric cancer (Evans *et al.*, 1998; Basso *et al.*, 1998). VacA allelic variation has been observed in *H. pylori* isolates from diverse geographical locations (Letley *et al.*, 1999; Ito *et al.*, 1998).

H. pylori VacA binds to target cells and is slowly internalised (Garner and Cover, 1996) into the cytoplasm, where its biological activity is expressed (de Bernard *et al.*, 1998). The interaction of VacA with target cells is mediated through high-affinity receptors

shown to be necessary for its biological activity (Massari *et al.*, 1998). The cytotoxin has been shown to be directly responsible for gastric epithelial injury by inducing intracellular vacuolation of cells (reviewed in Montecucco *et al.*, 1999) and these vacuoles are autophagasomal in nature (Harris *et al.*, 1996). In addition, VacA directly inhibits gastric epithelial cell proliferation, one of the main processes involved in gastric mucosal healing (Ricci *et al.*, 1996) and administration of the purified toxin to mice induces gastric mucosal lesions (Telford *et al.*, 1994).

Some *H. pylori* strains also express a protein of 120 kDa, which has been shown to be immunogenic in humans infected with *H. pylori* (Crabtree *et al.*, 1991). This antigen is expressed by the majority of VacA producing isolates, and consequently the gene was designated CagA (cytotoxin-associated gene) (Tummuru *et al.*, 1993) but is not required for expression of vacuolating toxin activity (Tummuru *et al.*, 1994). CagA is present in 88-100% of *H. pylori* isolates from patients with duodenal ulceration compared with 50-60% from patients with superficial gastritis alone (Crabtree *et al.*, 1991; Covacci *et al.*, 1993; Cover *et al.*, 1995). CagA+ strains induce higher levels of cytokine production than CagA- strains (Crabtree *et al.*, 1995) but this activity is not due to the CagA protein itself, as isogenic *cag*A- mutants retain the activity (Censini *et al.*, 1996). A region upstream of *cag* A has since been implicated in the enhanced virulence associated with CagA+ strains (Censini *et al.*, 1996). Several characteristics of this locus, such as the presence of short repeated sequences and insertion sequences, its G+C content (35%) (which is different to that of the genome (39%)), its prevalence in the most virulent strains and its capacity to encode proteins possibly involved in the export of virulence determinants (Censini *et al.*, 1996, see also section 1.9.2) has led to the consideration that this locus is a so-called pathogenicity island (Hacker *et al.*, 1997; Covacci *et al.*, 1997). The locus encodes a region of approximately 40 kilo bases (kb) and is referred to as the Cag pathogenicity island (cag PAI) (Censini *et al.*, 1996). *H. pylori* strains containing the cag PAI are more virulent than those that do not contain it, and are more often associated with peptic ulcer (Censini *et al.*, 1996) and gastric cancer (Blaser *et al.*, 1995; Crabtree *et al.*, 1995) although a recent study suggested that the presence of the cag PAI cannot reliably predict the outcome of infection (Jenks *et al.*, 1998).

H. pylori isolates have therefore been divided into two categories; Type I and Type II. Type I isolates contain the *cag* A gene and produce both the CagA and VacA proteins. Type II isolates, on the other hand, do not contain the *cag* A gene and do not produce either CagA or VacA (Xiang *et al.*, 1995). Although some intermediate phenotypes exist, Type I strains have been associated with more severe disease. A novel gene, recently identified and designated *ice*A (induced by contact with epithelium) (Peek *et al.*, 1998) may also be a major virulent determinant of *H. pylori.* Although the clinical significance of *ice*A is currently unknown, expression is up-regulated by contact between *H. pylori* and epithelial cells, and a recent study confirmed an association between *ice*A and ulcer disease (van Doorn *et al.*, 1998).

1.5 Diagnosis and treatment

1.5.1 Diagnosis of *Helicobacter pylori* infection

It was initially reported in 1985 that eradication of *H. pylori* diminished ulcer recurrence rates (Goodwin *et al.*, 1986). Since then, many advances have been made in both the diagnosis and treatment of the bacterium and a variety of tests are now available to diagnose *H· pylori* infection (reviewed in Dunn *et al.*, 1997). Bacterial culture and histological examination of gastric tissue obtained through endoscopy is the gold standard of diagnosis. Due to the cost and time constraints of culture and histology, the urease biopsy test is a quick and inexpensive alternative for diagnosis. Noninvasive tests used to diagnose *H. pylori* infection generally have good sensitivity and specificity compared with histological demonstration. Serology has proven to be a excellent noninvasive method of diagnosis and a number of accurate enzyme-linked immunosorbent assay tests are available that provide precise and quick diagnosis. The urease breath test (UBT) is recommended for diagnosis after treatment, as serum anti-*H. pylori* antibodies can persist for several months following eradication of the bacterium. Urease breath tests are, however, limited by the availability of sophisticated instrumentation. PCR may also be useful to diagnose *H. pylori* with a high degree of specificity and sensitivity. PCR has been used to show *H. pylori* in dental plaques, feces and gastric juice (Dunn *et al.*, 1997) and can be advantageous in distinguishing between reinfection versus recurrence of infection.

1.5.2 Treatment of *Helicobacter pylori* infection

The publication of the National Institutes of Health Consensus Development Conference guidelines on management of *H. pylori* infection in 1994 set a precedent (NIH, 1994). It is now universally accepted that patients with *H. pylori* infection and uncomplicated ulcer disease should receive eradication therapy (Vaira *et al.*, 1998). Therapeutic regimens currently used for eradication of *H. pylori* include both dual and triple therapies. Recent evidence suggests that the best treatment regimens include an acid-suppressant combined with an antibiotic such as amoxicillin given twice a day for two weeks, with or without a second antibiotic (reviewed in Axon, 1998).

Eradication of *H. pylori* leads to a rapid, complete disappearance of neutrophils from the gastric mucosa. The number of lymphocytes and plasma cells also decreases, but at a much slower rate (Kuipers, 1997). The slow disappearance of the mononuclear cell infiltrate is paralleled by a reorganisation of the mucosal structure, the reappearance of glands, and a slow return to normal acid production. Complete regression of the ulcer occurs in approximately 80% of treated individuals. Eradication *of H. pylori* leaves the patient with about a 2% risk of the ulcer recurring versus at least a 50% chance that the ulcer will recur over the next year if *H. pylori* is not eradicated (Matisko and Thomson, 1995).

The increased use of antibiotics in general and the lack of new compounds in recent years has resulted in an increased prevalence of bacterial resistance and treatment failures with many common infections, and *H. pylori* infection is no exception. Few antibiotics have proven effective in curing *H. pylori* infection, and the bacteria can acquire resistance to these compounds (reviewed in Goodwin, 1997 and Megraud, 1998a). Resistance to clarithromycin has been found in up to 15% of strains in some parts of the world, jeopardising its clinical efficacy. Resistance to metronidazole is even more widespread (Megraud, 1998b). In addition, antimicrobial therapies continue to have a number of limitations, including poor compliance and adverse side effects. The high cost of antimicrobial therapy is also prohibitive in many developing countries where *H. pylori* infection is endemic. Immunisation against *H. pylori* infection may therefore be a more viable approach to the management of *H. pylori*-related diseases (Ferrero and Labigne, 1996). Of key importance in the development of a vaccine

against *H. pylori* infection is the availability of suitable animal models to test the relevant virulence factors for their role in the pathogenesis of infection and to evaluate their ability to induce protective immunity for their consideration as candidate vaccine components.

1.6 *Helicobacter* infection in animals

1.6.1 Natural infection

The successful culture of *H. pylori* stimulated interest in stomach microbiology and in the search for a suitable animal model of *Helicobacter* infection, numerous studies ensued to determine whether gastric ulcer disease occurred naturally in animals. As a result, the genus *Helicobacter* has expanded rapidly in recent years to include organisms that inhabit the gastric mucosa of a number of animal species ranging from ferrets to cheetahs (Fox and Lee, 1996). With a few exceptions (see below), the host range of naturally occurring *H. pylori* infection is restricted to humans and non-human primates. Other species of *Helicobacter* that tend to be host-specific include *H. acinonyx*, which was originally isolated from captive cheetahs with chronic vomiting (Eaton *et al.*, 1993) and recently from the gastric mucosa of Sumatran tigers with chronic gastritis (Schroder *et al.*, 1998), *H. mustelae*, which naturally infects ferrets (Fox *et al.*, 1990) and *H. nemestrinae,* isolated from the pigtailed macaque (Bronsdon and Schoenknecht, 1988). Several species of non-human primates develop chronic gastritis in response to natural infection with *H. pylori* (Dubois *et al.*, 1994; Handt *et al.*, 1997) and are commonly colonised with large gastric organisms given the provisional name *H. heilmannii* (Solnick *et al.*, 1993). *H. heilmannii* was recently cultured from humans (Andersen *et al.*, 1999) and is associated with chronic gastritis, although less than 0.07-1.0% of the community are infected with this species (Heilmann and Borchard, 1991). In addition, a novel intestinal *Helicobacter* was recently identified from cotton-top Tamarins with chronic colitis and is the first to be identified in New World primates (Saunders *et al.*, 1999).

Domestic pets such as dogs and cats have been found to harbour a variety of spiral *Helicobacters* in their gastric mucosa (Eaton *et al.*, 1996). The main gastric *Helicobacter* species found in these animals are *H. heilmannii* (also known as *H.*

bizzozeroni) and *H. felis* (Neiger *et al.*, 1998, Eaton *et al.*, 1996) but the clinical significance and ecology of these species in domestic animals remains unclear (Jalava *et al.*, 1998; Lecoindre *et al.*, 1997). In cats and dogs, *H. heilmannii* has been associated with mild to moderate gastritis (Dieterich *et al.*, 1998, Eaton *et al.*, 1996), while others have reported little or no gastritis (Norris *et al.*, 1999). *H. heilmannii* is also commonly found in swine and has been associated with gastric ulcer disease in these animals (Queiroz *et al.*, 1996). *H. canis*, implicated in hepatitis in the dog (Fox *et al.*, 1996), was recently recovered from a kitten with severe diarrhea (Foley *et al.*, 1998), and may be of pathological significance. *H. pylori* infection has been associated with moderate to severe gastritis in domestic cats (Handt *et al.*, 1995, Fox *et al.*, 1996), but other studies have demonstrated that cats are unlikely to be a zoonotic source of *H. pylori* infection in man (El-Zaatari *et al.*, 1997). Finally, four *Helicobacter* species (*H. muridarum, H. rappini, H. hepaticus* and *H. bilis*) have been identified in the gastrointestinal tract of rodents (Mahler *et al.*, 1998) and one of these (*H. hepaticus*) has been associated with chronic active hepatitis and liver tumours (Fox *et al.*, 1996).

1.6.2 Animal models

In general, animal models of *H. pylori* infection have been limited by their relatively poor resemblance to human infections, notably because of the lack of an acute neutrophil response in the mucosa, and peptic ulcers are rarely a consequence of infection. Despite this, a number of models exist that have provided valuable information on *Helicobacter* infection and associated pathology (reviewed in Lee, 1998).

Germ-free piglets are susceptible to colonisation by *H. pylori* of human origin, and colonisation results in a chronic gastritis characterised by lymphocytes and plasma cells in the gastric mucosa (Krakowka *et al.*, 1991; Eaton and Krakowka, 1992). The piglet model has been most useful for studies of putative bacterial colonisation factors such as urease and flagellum (Eaton *et al.*, 1996). Many different species of monkeys have also been used to study gastric *Helicobacter* species (Shuto *et al.*, 1993; Bronsdon and Schoenknecht, 1988) since many are naturally infected with gastric bacteria, including *H. pylori.* Of the species studies, rhesus monkeys appear the most promising because of their worldwide availability, moderate size and the large repertoire of immunological

reagents available. In addition, the *H. pylori*-infected rhesus monkey exhibits atrophy, microerosions and loss of mucus reminiscent of that seen in humans (Dubois *et al.*, 1994) and was recently used for immunisation studies (Dubois *et al.*, 1998). *H. pylori*-induced gastritis has also been successfully induced in specific pathogen free cats (Fox *et al.*, 1995), conventional beagle dogs (Rossi *et al.*, 1999) and guinea pigs (Sturegard et al., 1998; Shomer *et al.*, 1998).

Ferrets are naturally infected with *H. mustelae* (Fox *et al.*, 1990) but there have been no previous studies exploring the question of prophylactic vaccination in this model, partly due to the almost universal colonisation of ferrets with *H. mustelae*. In addition, *H. mustelae* is genotypically and phenotypically different to *H. pylori* (Morgan *et al.*, 1991) and the pattern of colonisation of this organism differs from that observed with *H. pylori*-infected humans (O'Rourke *et al.*, 1992). Despite this, experiments with flagellar mutants have provided insights into the importance of motility in colonisation (Andrutis *et al.*, 1997). Recent advances have been made in *H. pylori* research using the Mongolian gerbil as a model (Matsumoto *et al.*, 1997; Wirth *et al.*, 1998). This small animal model represents one of the few models in which infection reproduces the ulceration observed in humans, and more importantly, it was recently demonstrated that long-term infection with *H. pylori* in the Mongolian gerbil induces gastric adenocarcinoma (Watanabe *et al.*, 1998).

Early reports suggested that mice were either not susceptible to colonisation with *H. pylori* or that colonisation was sporadic and not associated with gastritis (Karita *et al.*, 1991; Marchetti *et al.*, 1995). The discovery that mice were susceptible to infection with *H. felis* and that infection resulted in an active/chronic gastritis that mimicked the human pathology (Lee *et al.*, 1990, 1993), set a precedent for the use of this model in numerous studies investigating the pathogenesis of infection, immune response and for evaluation of vaccination protocols (see sections 1.7 and 1.8). The level of gastritis appears to be host dependent, with high colonisation rates achieved particularly in C57BL/6 mice, resulting in a gastritis resembling the human disease, with some neutrophils evident (Mohammadi *et al.*, 1996). It was recently shown that some strains of *H. pylori* can also be adapted to colonisation of mice (Lee *et al.*, 1997) and specific pathogen free mice (Ferrero *et al.*, 1998). Use of this standardised model is now widespread. The severe gastritis observed in recipient SCID mice also represents an

important addition to the growing number of animal models of disease (Eaton *et al.*, 1999). Using a variety of the available animal models of *Helicobacter* infection, the feasibility of developing both a prophylactic and therapeutic oral vaccine against *H. pylori* infection has been demonstrated. The induction of specific antibodies at mucosal sites is most efficiently achieved by local stimulation, therefore an oral vaccine is likely to be most effective. An understanding of the immune response to *Helicobacter* infection is an important prerequisite for the development of an effective oral *H. pylori* vaccine and will be discussed in the next section in the context of both human and animal results.

1.7 The immune response to *Helicobacter* infection

H. pylori differs from classical mucosal pathogens in that it does not invade host tissues, but rather inhabits a unique niche in the mucus overlaying the gastric epithelium. In this ecological niche, the bacteria are protected from the many components of the host's immune system. Despite this, the host mounts a vigorous immune response to *H. pylori* during infection, which generally fails to resolve the infection and may in fact contribute to disease. The key to understanding these immune responses lies in the complex interplay between the innate and acquired immune systems which combine to produce a potent integrated response in an attempt to eliminate the organism.

1.7.1 Innate immunity

Bacteria entering the gastrointestinal tract can be removed by a number of local nonspecific host defense mechanisms. Mucus lining the inner surfaces of the body acts as a protective barrier to prevent bacterial adherence to underlying epithelial cells (Phalipon and Sansonetti, 1999). Bacteria trapped within the mucus can then be removed by peristalsis and turnover of epithelial cells and the mucus layer. Bactericidal components of body fluids such as acid in the stomach gastric juice are also effective in preventing colonisation by microorganisms. However, the virulence factors of *H. pylori* (see section 1.4) have enabled this bacterium to colonise and persist in the hostile environment of the stomach where it initiates a complex and vigorous host immune response. Although generally ineffective at clearing the infection, the host immune response may eliminate *H. pylori* in some instances (Radcliff *et al.*, 1999).

The vertebrate immune response to infection can be separated into two distinct, but integrated immune systems; non-specific (innate) and specific (acquired/adaptive) (Lo *et al.*, 1999). Both systems play an important role in the defense against *Helicobacter* infection. Elements of innate immunity include an effective array of host phagocytic cells such as polymorphonuclear (PMN) neutrophils and macrophages, in addition to eosinophils and natural killer cells. Soluble factors such as complement and numerous acute phase proteins are also indispensable lines of defense. These factors combined enable the host to effectively distinguish between innocuous and potentially noxious substances according to their carbohydrate signatures. Recognition of carbohydrate structures occurs via receptors on host cells such as surface epithelial cells and the effector cells of the innate immune system. The receptor specificity is germline encoded and may have evolved because particular carbohydrate structures such as LPS are common constituents of bacterial cell walls and have structures distinct from eukaryotic cell surfaces (Fearon and Locksley, 1996).

Recognition of invading pathogens in this way results in the induction of signals that activate the components of the innate immune system. Chronic *H. pylori* infection is characterised by an infiltratration of polymorphonuclear (PMN) and mononuclear cells into the epithelium and underlying lamina propria (Figure 1.1). Neutrophils are an important component of the inflammatory response induced by *H. pylori* infection (Crabtree *et al.*, 1993). Since *H. pylori* is a non-invasive bacteria, it has been proposed that protein components and secreted products of the bacterium traversing the epithelial barrier may, by direct or indirect action on leukocytes, lead to their activation and enhanced local migration into mucosal tissue. *H. pylori* components with chemotactic activity for monocytes and neutrophils (Mooney *et al.*, 1991; Craig *et al.*, 1992; Broom *et al.*, 1992) have been found in the lamina propria of infected patients (Mai *et al.*, 1992). Activation of PMNs along with enhanced synthesis of various cytokines and increased secretion of reactive and monocytes results in increased expression of the surface molecule HLA-DR, oxygen metabolites (ROMs) (Mai *et al.*, 1991; Nielsen and Anderson, 1992b). *H. pylori* produces antiproteases that confer resistance to proteolytic attack by invading neutrophils (Takemura *et al.*, 1996). ROMS are highly toxic and can cause damage to cellular proteins, carbohydrates and DNA, however, *H. pylori* appears resistant to ROM toxicity, possibly through the production of superoxide dismutase (Spiegelhalder *et al.*, 1993; Gotz *et al.*, 1996). Release of ROMs and other

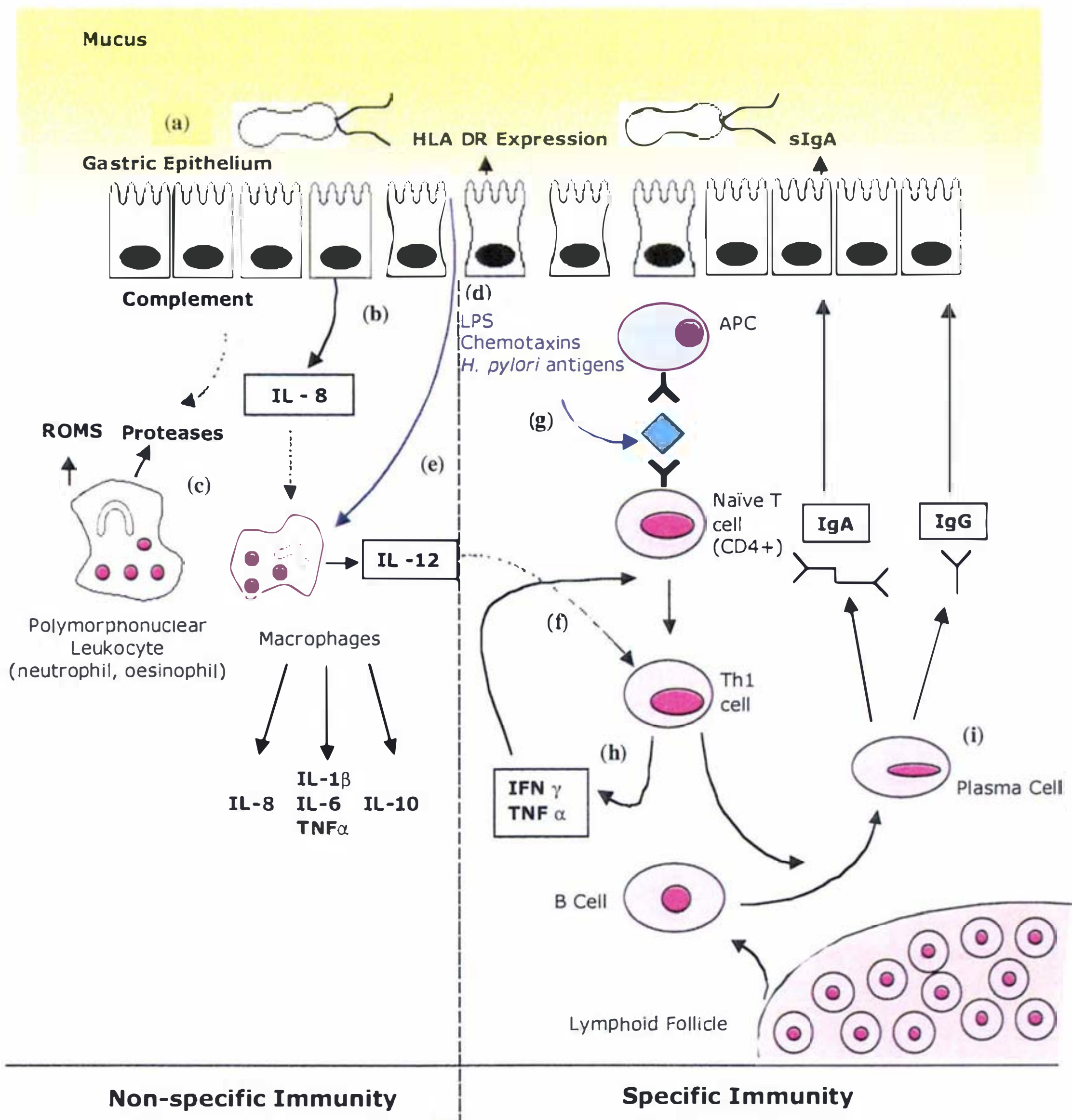


Figure 1.1: Schematic representation of the host immune response to *H. pylori* infection. (a) *H. pylori* infection leads to the presence of bacteria in the mucus of the gastric lumen (b) Upon contact with the epithelium, PAI containing bacteria induce the epithelial cells, through signaling pathways, to synthesise and release IL-8 (c) IL-8 is strongly chemotactic for PMN cells which release damaging ROMS and proteases in an attempt to kill the bacteria (d) Contact with the epithelium also leads to the release of *H. pylori* virulence factors eg. VacA, which induce epithelial damage (e) *H. pylori* antigens that cross the epithelial layer are able to activate macrophages to release several pro-inflammatory cytokines such as IL-8, IL-6, IL-1β and possibly IL-12 (f) IL-12 is important for polarising the CD4+ T helper response to a predominant Th1 phenotype (g) *H. pylori* antigens may also drive an antigen-specific Th1 response at the site of infection (h) Th1 cells release pro-inflammatory cytokines such as IFN-γ and TNF-α which may contribute to the maintenance of gastritis (i) B cells differentiate into plasma cells and produce both local IgG and sIgA antibodies at the site of infection (adapted from Telford *et al*., 1997).

proteolytic enzymes such as myeloperoxidase (Noorgaard *et al*., 1995) from stimulated PMN cells may then contribute to tissue damage characteristic of chronic gastric inflammatory diseases (Nielsen and Anderson, 1992a; Hansen *et al*., 1999; Davies *et al*., 1994). This theory is supported by the finding that *H. pylori* strains capable of activating neutrophils are found more often in peptic ulcer patients than in those with chronic gastritis (Rautelin *et al*., 1993; Nielsen and Anderson, 1995) and these strains are associated with more severe inflammation (Rautelin *et al*., 1996). In addition, *H. pylori* components induce nitric oxide synthase production by macrophages which may also contribute to inflammation and possibly malignant transformation in the gastric mucosa (Wilson *et al*., 1996).

Stimulation of leukocytes also induces the upregulation and activation of integrins, which enable the leukocytes to adhere to the endothelial cells of the vessel wall before migrating into tissues (Baggiolini, 1998). Upregulation of the β_2-integrin CD11b/CD18 glycoprotein complex expressed by activated neutrophils has been shown to be an important mediator of neutrophil adhesion to endothelial cells in *H. pylori*-induced inflammation (Yoshida *et al*., 1993; Enders *et al*., 1995). However, others have found no difference in the β_2-integrin upregulation on neutrophils and monocytes between *H. pylori* strains from ulcer patients and those from asymptomatic patients (Hansen *et al*., 1999). The ability of *H. pylori* products to attract and activate monocytes has been attributed to *H. pylori* products such as neutrophil-activating protein (HP-NAP) (Evans *et al*., 1995), urease (Harris *et al*., 1996), porins (Tufano *et al*., 1994) and LPS (Perez-Perez *et al*., 1995). The LPS of *H. pylori*, although capable of activating macrophages, exhibits much lower biological activity compared to other gram-negative bacteria (Perez-Perez *et al*., 1995) and may represent a natural selection for strains that do not evoke a detrimental strong inflammatory response (Kirkland *et al*., 1997). Studies also show that *H. pylori* whole bacteria evoke a strong non-MHC restricted cellular cytotoxic response by NK cells (Tarkkanen *et al*., 1993). Following the recruitment of granulocytes and monocytes to the site of infection, proinflammatory or immunoregulatory cytokines are released from these cells. The release of these cytokines induces the acute phase response, enhancing the microbial functions of the host's immune cells to *H. pylori*, resulting in the recruitment of more PMN cells and monocytes/macrophages (Genta, 1997).

The local production of cytokines plays an important role in the regulation of the PMN cell responses to *H. pylori* infection. *H. pylori* urease and recombinant urease is capable of activating mucosal macrophages and stimulating them to produce IL-1β, IL-6, IL-8 and tumor necrosis factor–alpha (TNF-α) (Harris *et al.*, 1996; Harris *et al.*, 1998). Mucosal biopsies from *H. pylori* infected patients also show significantly higher levels of IL-1β, IL-6, TNF-α and IL-8 (Crabtree *et al.*, 1991). TNF-α and IL-8 exhibit chemtoactic and proinflammatory activities that amplify the recruitment and activation of PMN cells (Mai *et al.*, 1992) as well as T cell chemotaxis. Gastric mucosal tissue from *H. pylori* infected individuals synthesizes IL-8 (Crabtree *et al.*, 1994) and studies using gastric epithelial cell lines showed upregulation of IL-8 expression in *H. pylori*-stimulated cells (Sharma *et al.*, 1995). In addition, live bacteria stimulate IL-8 expression in gastric epithelial cells to a greater degree than do killed bacteria, indicating that cellular components produced by live *H. pylori* are required for the induction of cytokine production by the epithelial mucosa (Crowe *et al.*, 1995, Huang *et al.*, 1995).

Strains of *H. pylori* that harbour the cag PAI (section 1.4.2) (CagA+) induce higher levels of IL-8 production (Crabtree *et al.*, 1995; Huang *et al.*, 1995; Yamaoka *et al.*, 1996). A number of *H. pylori* gene products encoded in this PAI, including the *pic*B gene product, are required for IL-8 expression, since mutations in these genes abrogates this IL-8 response (Tummuru *et al.*, 1995; Censini *et al.*, 1996). Recently, the human transcription factor, nuclear factor-kappa B (NF-kB), which regulates transcription of genes encoding cytokines such as IL-1, 6, 8 and TNF-α, was shown to be activated by exposure of gastric epithelial cells to *H. pylori* (Munzenmaier *et al.*, 1997; Masamune *et al.*, 1999) and this correlated with the ability of *H. pylori* to elicit IL-8 induction (Sharma *et al.*, 1998). An alternative pathway for IL-8 induction may also occur via *H. pylori* binding to gastric epithelial cells and induction of tyrosine phosphorylation of host cell proteins adjacent to the site of bacterial adherence (Segal *et al.*, 1997). More recently, proteins encoded by genes in the cag PAI were shown to be necessary for NF-kB activation (Glocker *et al.*, 1998) and also tyrosine kinase-dependent transcription of IL-8 in gastric epithelial cells (Li *et al.*, 1999). It has been proposed that these PAI encoded proteins form a surface structure which acts as the NF-kB-inducing agent (Glocker *et al.*, 1998). Indeed, other studies have shown that direct contact of *H. pylori*

with epithelial cells is a possible prerequisite for stimulation of IL-8 secretion and requires an intact bacterial membrane conformation (Reider *et al.*, 1997).

A consequence of the release of cytokines is the induction of inflammation and disruption of epithelial cell junctions which favours translocation of the bacteria across the mucosal barrier of the stomach (China and Goffaux, 1999). Gastritis can occur in the absence of T and B cells following infection of immunodeficient mice with *Helicobacter felis* (Blanchard *et al.*, 1995) suggesting that at least some of the changes in patients infected with *H. pylori* result from these nonspecific inflammatory responses. However, more recent reports have now shown that several mouse strains, including the strain used in above study, exhibit only mild gastritis following *Helicobacter* infection anyway (Roth *et al.*, 1999; Eaton *et al.*, 1999). Nevertheless, recent studies suggest that mucosal cytokine responses are responsible for the acute inflammatory response following infection, and play an important role in preventing colonisation by mucosal pathogens (Svanborg *et al.*, 1999). During this early phase, the immune system attempts to eliminate and control infection using innate inflammatory effector cells (Muraille and Leo, 1998). Signals provided by the components of the innate immune system then provide instructions to enable the acquired immune response to select appropriate antigens and strategies for bacterial elimination (Ogra *et al.*, 1997).

1.7.2 Acquired immunity

In addition to phagocytic cells, the presence of T lymphocytes and plasma cells in the inflammatory infiltrate in *H. pylori* gastritis indicates that antigen-specific cellular and antibody-mediated immune mechanisms are important. Acquired or adaptive immunity is mediated by T and B lymphocytes. In contrast to those of the innate immune cells, the specificities of lymphocyte receptors are generated by somatic mechanisms which results in a flexible, almost infinitely adaptable system (Fearon and Locksley, 1996). Recent studies using immunodeficient mice lacking B or T cells have demonstrated a crucial role for the acquired immune response to *Helicobacter* infection in the development of gastric epithelial pathology (Roth *et al.*, 1999; Eaton *et al.,* 1999). Understanding the nature of these responses is likely to aid in the development of an effective vaccine against *H. pylori* infection.

1.7.2.1 Cell-mediated immunity

Intestinal epithelium contain microfold epithelial (M) cells that are specialised in the delivery of material from the lumen to the underlying antigen-presenting cells (APC). Presentation of antigen to T cells occurs within mucosa-associated lymphoid follicles, such as the Peyer's Patches (PP). Stimulated T and B lymphocytes exit the PP and seed other areas of the intestine and mucosal tissues (Kaiserlian, 1999). This model implies that lymphocytes from PP also seed the stomach, however there is no direct evidence that this occurs (Ernst *et al.*, 1997). The mechanism of antigen uptake in the absence of PP and M cells in the gastric mucosa is unknown and it is not clear if gastric T cells arise as a result of local expansion or recruitment from other inductive sites such as PP. *H. pylori* infection results in the development of organised gastric lymphoid follicles (see section 1.3) which decline after eradication of *H. pylori*, suggesting that specific *H. pylori* antigenic stimulation is required (Hussel *et al.,* 1993). Accessory cells in this lymphoid tissue may facilitate antigen delivery from the gastric lumen. The mechanism of initial antigen uptake from the gastric lumen is not well understood, but could involve passive absorption of soluble products, direct epithelial endocytosis of shed bacterial antigens or passage of antigen through disrupted epithelial tight junctions (Crabtree, 1996).

T lymphocytes can be broadly divided into two general classes which are distinguished by the presence of a surface glycoprotein, either CD8 or CD4. CD8+ T cells, or cytotoxic T lymphocytes (CTL), recognise epitopes presented on the cell surface by major histocompatablility complex (MHC) class I molecules, and recognition results in lysis of the target cell (Whitton, 1998). CD4+ T cells, or T helper (Th) lymphocytes, recognise epitopes presented on the surface by MHC class II molecules, and orchestrate the immune response by promoting antibody production by B cells, intracellular killing by macrophages and clonal expansion of CTLs (Fearon and Locksley, 1996). The accumulation of T cells in the gastric mucosa during infection with *H. pylori* indicates that T cells are activated *in vivo* (Ernst *et al.*, 1995). Although epithelial cells do not normally express HLA-DR (Type II MHC), chronic gastritis is associated with an increase in epithelial HLA-DR expression (Valnes *et al.*, 1990) and epithelial cells from *H. pylori* infected patients express B7-2 (Ye *et al.*, 1997). The interactions of B7-1 (CD80) or B7-2 (CD86) on APCs with CD28 on T cells is required for the activation of

T helper cells (Swain, 1999). Gastric epithelial cells therefore appear to be capable of internalising and processing antigen, as well as stimulating CD4+ T cells (Crowe *et al.*, 1996). IFN-γ, which induces the de-novo expression of MHC class II and co-expression of B-1 (CD80) and B7-2 (CD86) on gastric epithelial cells, enabling them to act as antigen-presenting cells (Kaiserlian, 1999), is known to be produced during *H. pylori* infection (Moss *et al.*, 1994; Karttunen *et al.*, 1995; Yamaoka *et al.*, 1996).

Peptides presented by MHC class I antigen presenting cells derive from proteins synthesised in the cell (endogenous), whereas peptides presented by class II APCs derive from proteins which have been taken up by the APC, for example, via phagocytosis (exogenous) (Nandi *et al.*, 1998). As most bacteria, including *H. pylori*, exist and replicate extracellularly, they do not effectively enter the class I pathway, and generally fail to induce an effective CD8+ T cell response. CD8+ T cells are therefore most effective in eradicating intracellular infections. CD4+ T helper cells play a pivotal role in antigen-specific immune responses to *H. pylori* during infection. When compared with normal mucosa, *H. pylori* infected samples have increased numbers of predominantly CD4+ T cells (Bamford *et al.*, 1998) An increase in CD4+, but not CD8+, T cells correlates with increasing grades of gastritis and density of colonisation (Hatz *et al.*, 1996). In addition, *H. pylori* specific CD4+ T lymphocyte clones have been obtained from peripheral blood and gastric biopsies of infected patients (Di Tommaso *et al.*, 1995; D'Elios *et al.*, 1997). The T cells infiltrating the lamina propria during *H. pylori* infection are predominantly of the CD45RO+ phenotype, a marker for antigen-committed cells, and correlate with enhanced HLA-DR expression on gastric epithelial cells (Valnes *et al.*, 1990).

By producing different cytokines, CD4+ T helper lymphocytes direct the most appropriate counteraction against invading bacteria (Daugelat and Kaufmann, 1996). Based on experiments in mice, T helper cells can be classified into two main types, Th1 and Th2, according to their cytokine profiles. Although this functional dichotomy may not be so clear-cut in humans, in general Th1 lymphocytes produce IFN-γ, TNF–α and IL-2, which promote cell-mediated immune responses, whereas Th2 lymphocytes produce IL-4, IL-5, IL-6 and IL-10 cytokines and are mainly responsible for B cell development and antibody production, including mucosal IgA responses (Lindholm *et*

al., 1998). Th1 and Th2 cells derive from a common precursor cell (Th0) and their differentiation is regulated by cytokines present in the local environment. The cytokines necessary for Th1 or Th2 differentiation are IL-12 and IL-4 respectively (Swain, 1999). Therefore, one of the most important mechanisms allowing for direct communication between the non-specific and specific immune responses is the local production of cytokines or chemokines (Lo *et al.*, 1999).

Current dogma suggests that ideally mucosal immune responses should select for a Th2 response, resulting in increased production of secretory immunoglobulin A (sIgA). Paradoxically, during *H. pylori* infection, Th1 responses appear to predominate (Ernst *et al.*, 1997). IFN-γ – producing cells are increased in number during gastritis, whereas IL-4 – secreting cells are not (Karttunen *et al.*, 1995) and *H. pylori* can stimulate the production of IFN-γ by lymphocytes isolated from gastric tissue (Fan *et al.*, 1994) and peripheral blood (Tarkannen *et al.*, 1993). T-cell clones isolated from biopsy samples taken from *H. pylori*-infected patients display a cytokine secretion profile typical of Th1 cells, producing IFN-γ and TNF-α, but not IL4 or IL5 (Bamford *et al.*, 1998; Sommer *et al.*, 1998; Lindholm *et al.*, 1998). D'Elios *et al.*, (1997) demonstrated that a large proportion of the CD4+ T cell clones generated from the gastric mucosa of ulcer patients were specific for *H. pylori* antigens and that most of them (80%) exhibited a Th1-like cytokine profile, characterised by high levels of IFN-γ and TNF-α. In contrast, T cell clones from patients with mild gastritis show a more balanced Th1/Th0 ratio. There was no evidence of a Th2 response in either case. IL-12 is thought to play an important role in host responses to bacterial infections by inducing the production of IFN-γ by natural killer (NK) cells and T cells, and in association with IFN-γ, promoting the differentiation of naïve Th cells into the Th1 phenotype (Muraille and Leo, 1998). Haeberle *et al.* (1997) demonstrated that live *H. pylori* selectively stimulated the induction of IL-12 and Th1 cells *in vitro* and a recent study demonstrated that IL-12 was increased in *H. pylori* infected individuals compared with non-infected patients with the same degree of gastritis (Bauditz *et al.*, 1999) indicating a pivotal role for this cytokine in gastric inflammation.

The presence of Th1-like cells in the gastric mucosa implies that cell-mediated immune responses are induced during infection with *H. pylori*. Given that *H. pylori* is an

extracellular infection, and that the biological niche of the organism is the gastric lumen, a cell-mediated immune response is unlikely to be protective. This notion is supported by the fact that *H. pylori* infection persists for the life of the host. Excessive cell-mediated immune responses in close proximity to the epithelium could perturb the function of gastric epithelial cells (Bamford *et al.*, 1998) and induce chronic inflammation through the production of IFN-γ, which has been shown to induce apoptosis in gastric epithelial cells (Fan *et al.*, 1998). Murine studies also show that a Th1 response is associated with gastric pathology (Mohammadi *et al.*, 1997; Roth *et al.*, 1999; Eaton *et al.*, 1999). Indeed, *in vivo* neutralisation of IFN-γ with an anti-IFN-γ monoclonal antibody significantly reduces gastritis in *H. felis* infected mice (Mohammadi *et al.*, 1996) (see also section 1.8).

In contrast to reports supporting the induction of cell-mediated immune (CMI) responses by *H. pylori*, early studies indicated that *H. pylori* may even suppress CMI responses, by down-regulating Th1 responses and enhancing the Th2 response (reviewed in Fan *et al.*, 1996). Predominant Th2 responses have previously been associated with persistence of chronic infections (Daugelat and Kaufmann, 1996), and is consistent with *H. pylori* being a "slow" bacterial pathogen (Blaser, 1993). *In vitro* studies have suggested that *H. pylori* possesses some immunosuppressive actions, which may enable the bacteria to either evade bacterial clearance or to avoid excessive inflammation-mediated damage to the host (Fan *et al.*, 1996). Reports have demonstrated that *H. pylori* inhibits mitogen-induced proliferation of peripheral blood mononuclear cells (PBMCs) (Knipp *et al.*, 1993; Birkholz *et al.*, 1993). *H. pylori* whole cell preparations induce lower proliferative responses by PBMCs from *H. pylori* infected versus non-infected patients (Karttunen *et al.*, 1991, 1995; Fan *et al.*, 1994; Sharma *et al.*, 1994; di Tommaso *et al.*, 1995; Chmiela *et al.*, 1996).

Increased levels of IL-10 have also been reported in *H. pylori* infected mucosa (Bodger *et al.*, 1997; Yamaoka *et al.*, 1997; Bauditz *et al.*, 1999). IL-10 is a potent inhibitor of phagocyte and lymphocyte responses and is produced by a variety of human cells, particularly activated macrophages (Henderson and Wilson, 1995). Decreased IFN-γ production by blood and gastric lymphocytes in *H. pylori* infected compared to non-infected individuals has also been demonstrated (Fan *et al.*, 1994), in addition to

increased IL-6 in supernatants from cultured PBMCs (Fan *et al.*, 1996) and gastric tissue (Crabtree *et al.*, 1991). *H. pylori* may also interfere with protective immunity by acting on APCs through the release of VacA, which has recently been show to impair antigen processing and therefore subsequent stimulation of CD4+ T cells (Molinari *et al.*, 1998). This immune down-regulation may limit tissue damage caused by inflammation, but may also contribute to the failure of the immune response to clear the infection (Bodger and Crabtree, 1998).

1.7.2.2 Antibody-mediated immunity

Although a Th1 response predominates, humoral immunity also plays a role in the immune defense against *H. pylori* infection. Gastric T cell clones express helper function for B cell proliferation and immunoglobulin secretion that is antigen-dependent (D'Elios *et al.*, 1997). A study examining gastric mucosal humoral responses found *H. pylori* specific B cells in infected but not non-infected individuals (Mattsson *et al.*, 1998). *H. pylori* specific IgA and IgM antibodies are detectable in gastric juice (Sobala *et al.*, 1991) and there is local production of *H. pylori* specific IgG and IgA (Crabtree *et al.*, 1991). IgA is the predominant humoral effector at mucosal surfaces and can mediate host defenses within luminal secretions, the epithelium and the lamina propria (Bouvet and Fischetti, 1999). In the mucosa, IgA molecules are predominantly produced as dimers, which are bound to secretory component (SC) and transported in endocytotic vesicles to the apical side of epithelial cells. Subsequent proteolytic cleavage of SC results in the release of secretory IgA (s-IgA) (Corthesy and Kraehenbuhl, 1999). Conflicting results regarding the presence of SC in the healthy stomach have been published, but an association between gastritis and increased expression of SC by gastric epithelial cells has been reported (Ahlstedt *et al.*, 1999). Mucosal IgA is likely to be important in inhibiting antigen uptake, blocking bacterial adherence and motility of the organism, and also in toxin neutralisation (Russell *et al.*, 1999).

Gastric IgA antibodies can inhibit the vacuolation of epithelial cells induced by the cytotoxin *in vitro* (Figura and Crabtree, 1994). In a study of the gastric mucosal IgA response to *H. pylori* infection, IgA antibodies were found to recognise several *H. pylori* antigens, in particular the 120 kDa antigen later described as the cytotoxin-

associated protein (CagA) (Crabtree *et al.*, 1991). Nevertheless, *H. pylori* infection can persist for the life of the host, indicating that IgA-related defense mechanisms are not effective in eradicating *H. pylori* infection. In a study of IgA-deficient individuals, lack of sIgA did not seem to have any major effect on the establishment of infection, arguing against a pivotal role of IgA in defense of *H. pylori* infection (Bogstedt *et al.*, 1996). In addition, a more recent study found no difference in SC expression or IgA positive cells between *H. pylori* asymptomatic and ulcer patients, implying that local sIgA does not influence the outcome of infection (Ahlstedt *et al.*, 1999). Under physiological conditions, IgA has poor or no complement activating activity (Russell *et al.*, 1999), but complement activation has recently been suggested as being important in *H. pylori* gastritis perhaps mediated through mucosal IgG antibodies (Berstad *et al.*, 1997).

Chronic infection with *H. pylori* is also associated with the development of specific local and systemic IgG antibody responses to the bacterium (Tinnert *et al.*, 1997). Serum IgG antibodies are important components of the humoral response of the host to *H. pylori* infection. Serum IgG antibodies from *H. pylori* infected individuals enhances complement-mediated phagocytosis of *H. pylori* by PMN cells (Tosi and Czinn, 1990). Immunohistochemistry has demonstrated *in situ* coating of *H. pylori* lining the epithelium with host antibodies (Wyatt *et al.*, 1986) and activated complement components (Berstad *et al.*, 1997), however the role of such antibodies *in vivo* is unclear (Wyatt *et al.*, 1986). Early *in vitro* studies suggested that *H. pylori* can activate the classic pathway of complement, even in the absence of antibody (Bernatowska *et al.*, 1989). The presence of activated C3b on *H. pylori* in the gastric mucosa indicates complement activation, but whether this involves the classical or alternative pathway has yet to be determined. The presence of antibody-coated *H. pylori* (Wyatt *et al.*, 1986) and the possible involvement of LPS in the initiation of the alternative pathway (Kuby, 1997), suggests that both complement activation pathways may be involved in *H. pylori* associated gastritis. Whether complement activation is involved in host defense mechanisms or whether it mediates the tissue damage associated with gastritis remains unclear (Ferrero *et al.*, 1997). Complement activation may contribute to the neutrophilic response in the gastric mucosa as the presence of activated C3b is significantly related to neutrophil infiltration *in vivo* (Berstad *et al.*, 1997). Urease also exhibits immunomodulatory functions by inhibiting opsonisation of *H. pylori* in the presence of low concentrations of *H. pylori* specific antibodies (Rokita *et al.*, 1998).

1.7.2.3 Autoimmunity

In addition to a specific local humoral response to the bacterium, infection with *H. pylori* also results in a humoral response against autoantigens. An autoimmune response has long been postulated to play a role in gastritis (reviewed in Appelmelk *et al.*, 1997). Serum samples from most infected patients specifically cross-react with the gastric mucosa, but not with other tissues (Negrini *et al.*, 1991). In addition, an *H. pylori* specific monoclonal antibody shown to be cross reactive with both human and murine gastric mucosa caused stomach pathology in mice (Negrini *et al.*, 1996). The O-polysaccharide chains of LPS from some *H. pylori* strains exhibit mimicry of Lewis x and Lewis y blood group antigens (Wang *et al.*, 1999) found in the gastric mucosa (Appelmelk *et al.*, 1996). The expression of Lewis antigens by *H. pylori* can induce an immune response when antibodies recognise gastric glycoprotein targets, thus causing autoimmune inflammation (Monteiro *et al.*, 1998). This autoimmune mechanism is thought to lead to cell injury and contribute to gastritis (Appelmelk *et al.*, 1997). Wirth *et al.*, (1997) have suggested that the relative expression of Lewis x and Lewis y corresponds to the host Lewis phenotype, indicating selection for host-adapted strains. By analogy with mycobacterial and chlamydial heat shock proteins (HSPs), *H. pylori* HSPs may also contribute to gastric injury by stimulating T cells that cross react with similar determinants on the endogenous HSPs from stressed host cells. Antibodies against the heat shock protein HspB, found in infected patients, display cross-reactivity with the human homologue heat shock protein (Hsp60) (Kansau and Labigne, 1996). A potential role of HspB in the development of gastritis was also suggested in a study in which a monoclonal antibody raised against HspB also reacted with gastric epithelial cells, suggesting the presence of a common epitope (Yamaguchi *et al.*, 1997). Binding of T cells to MHC class II, B-1/B-7 and autoantigen presented by gastric epithelial cells in *H. pylori* gastritis, could lead to the activation and differentiation of the autoreactive B cells detected in *H. pylori* infected patients (Faller *et al.*, 1997; reviewed in Kirchner *et al.*, 1998).

1.8 Vaccination against *H. pylori* infection

The feasibility of a prophylactic vaccination against *Helicobacter* infection was initially demonstrated by experiments in which administration of *H. pylori* sonicates, together with mucosal adjuvant cholera toxin (CT) induced the production of specific serum and

gastrointestinal IgG and IgA (Czinn and Nedrud, 1991). Subsequent studies demonstrated that administration of *H. felis* sonicate given concurrently with CT protects mice against experimental infection with *H. felis* (Chen *et al.*, 1992, 1993; Lee and Chen, 1994) and protection was long-term (Radcliff *et al.*, 1996). In addition, the feasibility of a therapeutic immunisation against *H. felis* infection in mice was established when whole cell sonicate (Doidge *et al.*, 1994) or urease B subunit (Corthesy-Theulaz *et al.*, 1995), along with CT, was found to eradicate the bacteria in 50% of animals experimentally infected with *H. felis*. More recently, therapeutic immunisation with recombinant VacA or CagA against *H. pylori* infection in mice was demonstrated (Ghiara *et al.*, 1997). *H. pylori* urease alone is well tolerated in humans (Kreiss *et al.*, 1996) and administration of urease and *E. coli* heat-labile toxin (LT) to infected volunteers significantly decreases bacterial load but with no cure of infection. Therapeutic immunisation against *H. mustelae* in naturally infected ferrets has also been demonstrated (Cuenca *et al.*, 1996).

There is now much data available to support the development of a vaccine against *H. pylori* infection, and significant advances have been made in the identification of protective antigens, experimental adjuvants, delivery vehicles and the optimal route of administration. Only the most relevant advances in vaccine development are described below, as it is beyond the scope of this thesis to review all of the literature related to this topic, and a number of recent reviews are available (Blanchard *et al.*, 1999; Rijpkema, 1999; Vyas and Sihorkar, 1999; Kleanthous *et al.*, 1998). The mechanisms of protective immunity are discussed in more detail.

A number of purified or recombinant *H. pylori* antigens including the urease B subunit (Ferrero *et al.*, 1994; Michetti *et al.*, 1994), VacA (Marchetti *et al.*, 1995), catalase (Radcliff *et al.*, 1997) and GroES homolog (Ferrero *et al.*, 1995), have been shown to have protective properties in murine (Marchetti *et al.*, 1995) and non human primate (Dubois *et al.*, 1998) models of *H. pylori* infection. Oral immunisation with a combination of recombinant HspA and urease in mice achieved 100% protection against challenge with *H. felis* (Ferrero *et al.*, 1995). The administration of *H. pylori* antigens for protective immunisation requires a mucosal adjuvant such as CT or LT (reviewed in Elson and Dertzbaugh, 1999), but the toxicity of these precludes their use in humans. Recently, oral (Marchetti *et al.*, 1998) and intranasal (Kleanthous *et al.*,

1998) immunisation with purified *H. pylori* antigens (native and recombinant VacA, urease and CagA) co-administered with a non-toxic mutant of LT protected mice against *H. pylori* infection. Corthesy-Theulaz *et al.*, (1998) have used an attenuated strain of *Salmonella typhimurium* expressing *H. pylori* urease A and B subunits to achieve protective immunity against challenge with *H. pylori* in mice, and may represent an alternative approach to mucosal adjuvants. Systemic vaccination of mice with *H. pylori* antigens, using aluminum hydroxide as an adjuvant, protected mice against challenge with *H. pylori* (Nedrud *et al.*, 1999) demonstrating that an effective adjuvant already approved for use in humans can be used.

The induction of protective immunity against invading pathogens requires the stimulation of a specific type of immune response. As discussed above, the type of immune response mounted is dependent upon the local cytokine environment and the nature of the T-cell repertoire (Th1/Th2) induced. During infection with *H. pylori*, Th1 cells predominate (see section 1.7.2). Studies using the *H. felis* mouse model have also demonstrated the induction of Th1 phenotype in mice infected with *H. felis* (Mohammadi *et al.*, 1996). In contrast, mice immunised against *H. felis* appear to develop a Th2-type response (Mohammadi *et al.*, 1997; Saldinger *et al.*, 1998; van Doorn *et al.*, 1999). Observations supporting a role for Th2 based immunity to *Helicobacter* infection are consistent with the known activities of the mucosal adjuvants such as CT and LT used in the vaccination protocols (Blanchard *et al.*, 1999).

IgA responses have been shown to be selected by cytokines produced by Th2 cells. A shift in the T-cell response from Th1 to Th2 in *H. pylori* infection may thus favor the development of IgA responses and protective immunity. Mucosal IgA antibodies may play a role in protective immune mechanisms following immunisation against *Helicobacter* infection. Researchers have found increased levels of IgA concentrations in the secretions of immunised mice (Pappo *et al.*, 1995), but few studies have correlated this with protection. Support for a role of IgA in protection comes from evidence that IgA antibodies recognising urease are sufficient to provide protection in animal models (Lee *et al.*, 1995; Blanchard *et al.*, 1995). However, the mucosal IgA antibodies that occur in response to natural and experimental infection appear to be incapable of eliminating such infections (see section 1.4.2.2). Indeed, in some instances, the induction of protective immune responses in mice does not always

correlate with the production of mucosal IgA antibodies (Pappo *et al.*, 1995; Ferrero *et al.*, 1995) and studies using the *H. felis* mouse model show that IgA deficient mice can be protectively immunised (Nedrud *et al.*, 1996). Blanchard *et al.*, (1999) found that immunisation induced the production of local antibodies with antigenic specificities different from those induced by natural infection, suggesting that specificity, rather than the magnitude of antibody may be important.

Recent work using the Rhesus monkey as a model of *H. pylori* infection has also suggested that neither serum nor secretory IgA plays a protective role against infection (Dubois *et al.*, 1998), and has led to investigations into the role of IgG, which is generally neglected in mucosal immunity. Previous work has demonstrated that administration of IgG is sufficient to confer passive protection against GI infections (Robbins *et al.*, 1995). Support for a role of IgG in protective immunity comes from the recent finding that oral immunisation of mice with *H. pylori* sonicate or recombinant antigens, along with CT, results in the induction of specific IgG-secreting cells in the gastric mucosa. The proliferation of such antibody-secreting cells correlated with both the presence of IgG in gastric secretions and protection against *H. felis* infection (Ferrero *et al.*, 1997). In addition, the administration of the GroES homolog of *H. pylori* increased the production of specific anti-*H. pylori* IgG1 in mice and concurrently protected them against experimental *H. felis* infection (Ferrero *et al.*, 1995). A recent study investigating the role of IFN-γ in *H. pylori* infection concluded that this cytokine was not essential for protective immunity and played a more important role in the induction of gastric inflammation (Sawai *et al.*, 1999). Their data indicated that the protective immunity induced in mice by oral immunisation was based on humoral immunity, specifically serum IgG.

On the other hand, the observation that Th2-type cells can reduce the *H. felis* burden when adoptively transferred into unimmunised recipients (Mohammadi *et al.*, 1997) and the recent finding that MHC deficiency exacerbates *H. pylori* infection (Pappo *et al.*, 1999) implicates a role for cell-mediated immune responses in controlling the magnitude of infection. In addition, experiments in μMT antibody deficient mice have revealed that protective immunity can be achieved in the absence of any antibody

(Ermak *et al.*, 1998). Thus, the mechanism by which protective immunity to *Helicobacter* infection is mediated remains to be established.

It is worth noting that other species of animals appear less amenable than mice to protection by vaccination, for example, oral vaccination in species such as piglets (Eaton *et al.*, 1998) tends to suppress infection but not completely prevent it. Differences in the efficacy of vaccination against *Helicobacter* infection in other species is likely to be due to variability in vaccination protocols, host and bacterial factors (Dubois *et al.*, 1999). The identification *H. pylori* proteins and characterisation of their involvement in the immune response to infection is critical to developing an effective vaccine. The next section describes the mechanisms by which a gram-negative bacterium such as *H. pylori* may secrete virulence factors that interact with immune cells, and reviews some of the current knowledge on *H. pylori* exported proteins.

1.9 Secretion of bacterial virulence factors

1.9.1 Bacterial secretion systems

Secreted or surface-exposed bacterial proteins have long been known to play central roles in bacterial-host interactions. Proteins secreted by gram-negative bacteria, must pass through a number of compartments before they are expelled into the extracellular milieu. Gram-negative bacteria have an outer membrane situated above a thin peptidoglycan layer. Sandwiched between the outer membrane and the inner membrane cytoplasmic membrane), is a concentrated gel-like matrix (the periplasm) in the periplasmic space (Beveridge, 1999). Proteins synthesised in the cytoplasm at ribosomes must be targeted to the right compartment in order to fulfill their specific function. Three general secretion pathways have been described in gram-negative bacteria that export bacterial proteins (Figure 1.2), as well as a fourth pathway for group of bacterial proteins that mediate their own transport.

1.9.1.1 Type I secretion

In the Type I system, the protein is secreted in a one-step mechanism in which the protein crosses directly from the cytoplasm to the cell surface. Proteins transported by this system lack classic signal sequences and require three or four accessory molecules

that form a transmembrane channel through which the secreted protein moves. The type I secretory machinery consists of a homologue of an ABC transporter believed to be involved in providing the energy required for translocation. The rest of the apparatus includes an outer membrane component and a dimeric protein spanning the inner and outer membrane which belongs to a family of molecules referred to as membrane fusion proteins (Lory, 1998). The genes that encode these accessory molecules are usually found clustered with those that encode the secreted molecule. The carboxyl-terminal region of proteins secreted by this system contains the information necessary for secretion (Finlay and Falkow, 1997).

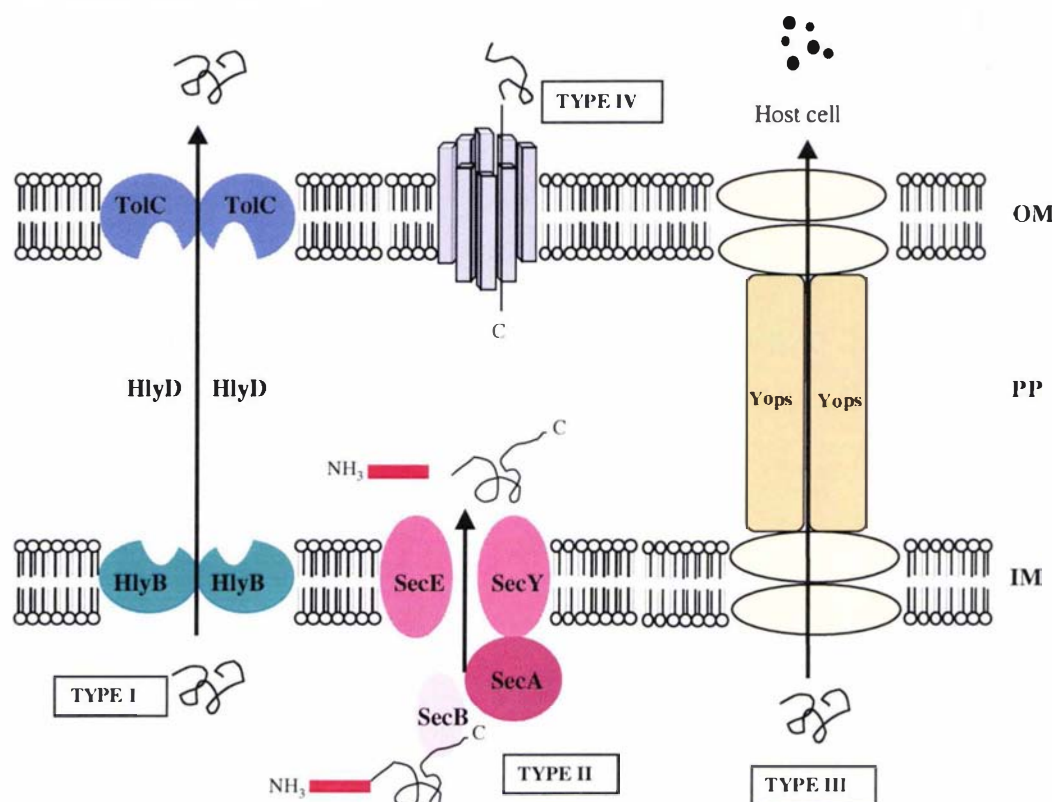


Figure 1.2: Schematic overview of the Type I, II, II and IV secretion systems for protein export in Gram-negative bacteria. The General Secretory Pathway (Type II) uses protein encoded signal peptides to direct export across the inner membrane, followed by cleavage of the signal sequence. Translocation across the outer membrane occurs in a second step via one of the main terminal branch (MTB) pathways. OM: outer membrane; PP: periplasm; IM: inner membrane. See text for further details.

This system is exemplified by the HlyA hemolysin of *E. coli*, an important virulence factor *in E. coli* extraintestinal infections. HlyA has an unprocessed export signal at its extreme C terminus specifically within the last 48 amino acids (China and Goffaux, 1999). HlyA is secreted through the inner and outer membrane via an oligomeric complex composed of a large ATP-binding cassette (ABC) transporter (HlyB) and

accessory factor (HlyD) and an additional outer membrane factor (TolC) (Figure 1.2). HlyB is assumed to be central to substrate recognition. The structure of the membrane translocator, and the generation of energy from ATP together with proton motive force, drives protein translocation. HlyD is suggested to be the membrane fusion protein as it may bring the protein in contact with the outer membrane channel. TolC forms a porin-like channel that creates a periplasmic bridge to the energized inner membrane components of the translocation complex (China and Goffaux, 1999).

1.9.1.2 Type II secretion – the General Secretory Pathway

The Type II secretion system is generally described as the classical system, general secretory pathway (GSP) or the sec-dependent secretion system. Type II secreted proteins use the GSP to reach the periplasm and then traverse the outer membrane through specialised secretory machinery consisting of at least 12 different components. This latter step is an extension of the GSP and is often referred to as the terminal branch. The type II secretion system is the major export pathway in most gram-negative bacteria (Finlay and Falkow, 1997).

Proteins transported by the GSP are distinguished from other proteins by the presence of a signal sequence. Signal sequences are characterised by a long, hydrophobic region (H domain) that is usually preceded by one or more positively charged residues in a short, generally hydrophilic region (the N domain). The C domain is usually less hydrophobic and contains the signals that are recognised by the signal peptidases (Perlman and Halvorson, 1983). The N region has been suggested to bind the negatively charged surface of the lipid bilayer of the membrane while the positive charges rare equired to orient the signal sequence of the secretory protein (or the stop-transfer signal of membrane proteins) correctly within the lipid layer (von Heijne, 1990). The H domain is the hydrophobic core of a signal sequence and varies in length from 7 to 15 amino acids. It is the most important part of the signal sequence and is responsible for positioning the C domain within reach of the signal peptidase (von Heijne, 1998). The signal peptidase site is the only part of the signal sequence that contains primary sequence specificity. Signal sequences are exclusively amino terminal, and although they all have essentially similar structural features, they can be divided into three types. The standard signal peptide as described above has three

domains with a cleavage site usually preceded by an Ala-X-Ala amino acid sequence, which is cleaved by signal peptidase I (Perlman and Halvorson, 1983). A small number of bacterial lipoprotein signal peptides are processed by signal peptidase II rather than signal peptidase I. A well-conserved consensus sequence Leu-Ala-(Gly, Ala)-Cys, defines the signal peptidase II cleavage site. Lipoprotein signal sequences lack a polar C-region, and are therefore usually shorter, and have more hydrophobic amino acids in the H region due to the nature of the cleavage site (von Heijne, 1990). Finally, type IV cleavage signal peptides are considered not to have a C domain since they are cleaved between the N and H domains (Pugsley 1993).

The *E. coli* Sec translocation machinery is an enzyme complex consisting of several integral membrane proteins and a peripheral bound ATPase. The core of this multisubunit enzyme is formed by the integral membrane proteins SecY, SecE and SecG, together with the peripherally bound SecA and are termed translocase (see Figure 1.2). Additionally, there are two membrane proteins, SecD and SecF, that are not essential for protein translocation but stabilise SecA in its active conformation. This Sec system is collectively responsible for the translocation of precursor protein across the cytoplasmic membrane and the insertion of integral membrane proteins into the membrane (Schatz and Beckwith, 1990). The first step in Type II secretion involves the targeting of the presecretory protein (preprotein) to the translocon in the cytoplasmic membrane. The targeting of the preprotein to the Sec apparatus for translocation is accomplished by several pathways of which the signal recognition particle (SRP)- and SecB-dependent targeting routes are the best studied. The choice of pathway is probably governed by the preprotein's signal sequence, by sequence motifs within the mature region of the precursor, and by the competition between folding and binding to SecB (Schatz and Dobberstein, 1996).

SecB-dependent targeting

SecB is a molecular chaperone that plays a dedicated role in guiding many precursor proteins to the cytoplasmic membrane. Although other chaperones such as GroEL DnaK and DnaJ are capable of maintaining a preprotein in a translocation-competent state *in vitro* (Langer *et al*., 1992), they fail to stimulate the translocation. This suggests that they can substitute for SecB in the translocation reaction with regard to interaction

with the preprotein, but cannot properly target the preprotein to the translocase. The processing of the SecB-independent precursor of alkaline phosphatase is disturbed in cells lacking DnaK or DnaJ, and it has been suggested that these chaperones play a more general role in the targeting of SecB-independent precursor proteins (Wild *et al.*, 1996). Although there are some conflicting opinions, it is generally believed that SecB binds the preprotein at the mature domain and that the signal sequence, although not directly involved in this binding, slows the folding process of the mature domain, allowing SecB to recognise only those proteins destined for translocation (Driessen *et al.*, 1998). SecB binding keeps preproteins in a translocation competent state and targets them to the SecA subunit of the membrane-bound translocase. The receptor for SecB at the membrane is SecA, and the binding site on SecA for SecB is located at the carboxyl terminus and is conserved among the bacterial SecA proteins. Upon interaction of SecB with SecA, SecB releases the precursor protein, which is subsequently transferred to SecA (Fekkes and Driessen, 1999). SecA binding of ATP then induces a conformational modification of SecA leading to its insertion into the inner membrane. The ATP hydrolysis induces the return to the initial conformation and liberation of the preprotein. This cycle is then repeated leading to a deeper insertion of SecA into the membrnae. Repeated cycles of SecA preprotein association, ATP-binding and hydrolysis, and SecA-preprotein dissociation result in the stepwise translocation of the entire preprotein across the membrane (Driessen *et al.*, 1998).

SRP-dependent targeting

Some proteins are exported independently of SecB, although interaction with the rest of the Sec export apparatus is required. Among these are alkaline phosphatase, ribose-binding protein, β-lactamase and the outer membrane lipoprotein (Wild *et al.*, 1992). In this system, proteins are targeted to the translocon by a signal-recognition particle (SRP) and SRP receptor. Targeting occurs cotranslationally with the proteins still attached to the ribosome. Directly after the signal sequence emerges from the ribosome, the nascent chain is bound by SRP. The *E. coli* SRP recognises and binds its substrates due to the presence of a highly hydrophobic signal sequence. This interaction results in a pause in the translation of the nascent chain and the ribosome-bound complex is then targeted to the SRP receptor. The interaction between SRP and its receptor releases SRP from the ribosome and the signal sequence. Subsequently the translation arrest is

relieved, the ribosome binds the membrane-embedded translocon and the preprotein is threaded across or into the membrane by chain elongation. The SRP of *E. coli* is comprised a 4.5S RNA molecule and a homologue of the 54 kDa eukaryotic SRP protein subunit called Ffh (fifty-four homologue). In *E. coli*, the SRP receptor, FtsY, is peripherally associated with the membrane and a membrane-bound FtsH homolog has been identified in *H. pylori* which may be involved in folding and assembly of proteins into and through the membrane (Ge and Taylor, 1996). At the membrane, SRP is released from the preprotein in a GTP-dependent manner. The preprotein, or integral membrane protein, is subsequently transferred to the translocase, which consists of the Sec apparatus (Fekkes and Driessen, 1999) and is exported across the membrane as described above.

Once a protein has traversed the inner membrane into the periplasm, a second step is then required to export the protein into or across the outer membrane if required. This is most often achieved by the main terminal branch (MTB) of the GSP, which is used for the export of a wide variety of extracellular proteins, and requires at least 14 accessory proteins in order to be functional (Pugsley *et al*., 1997). One representative of the MTB is the pullulanase (PulA) secretion pathway of *Klebsiella oxytoca* which has been extensively studied (Pugsley, 1993). Despite this, little tangible progress has been made towards identifying MTB components that determine secretion specificity or which form the putative channel through which secreted proteins cross the outer membrane. It is generally accepted that once they reach the periplasm, secreted proteins adopt tertiary and even quaternary structure before they are recognised by the components of the MTB and transported across the membrane. This secretion-competent conformation may provide the signal required for MTB secretion or may present secretion sequence signals in a way that is recognised by the transport system (Pugsley *et al*., 1997).

In addition to the MTB, a well characterised chaperone/usher assembly pathway is required for the assembly of over 30 adhesive organelles in a wide range of Gram-negative pathogenic bacteria (Thanassi *et al*., 1998). In particular, the assembly of P-pili by this pathway has enabled the investigation of the molecular mechanisms involved in the translocation of these proteins across the outer membrane (OM). The process involves a periplasmic chaperone (PapD) that binds to the pilus subunits as they

enter the periplasm, capping their interactive surfaces. These chaperone-subunit complexes are targeted to the OM usher assembly sites. The usher (PapC) is needed for the translocation of the subunits across the OM and is thought to form a pore via their largely β-sheet secondary structure, which is typical of bacterial OM pore-forming proteins. The next chaperone-subunit complex to reach the usher causes the displacement of the chaperone which results in the exposure of the subunit interactive surface allowing it to interact with the incoming subunit. Subunit-subunit interactions at the usher lead to the formation of linear pilus fibers which are then translocated through the pore and form into a final helical formation on the outside of the cell (Thanassi *et al.*, 1998). Unlike the MTB secretion system, the chaperone/usher pathway requires only two helper proteins and appears to be energy-dependent.

1.9.1.3 Type III secretion

Like the type I secretion pathway, type III secretion is independent of the sec system. Type III secretion systems, which are found in various gram-negative organisms, are specialised for the export of virulence factors delivered directly to host cells (Anderson and Schneewind, 1999). Each of the pathways comprises a complex apparatus of more than 20 proteins that assembles into a highly regulated channel through the inner and outer membranes (Henderson *et al.*, 1998). Interestingly, many of the inner membrane proteins are homologous to components of the flagellar biosynthesis apparatus of both Gram-negative and Gram-positive bacteria (Finlay and Falkow, 1997). This homology suggests that the two systems are functionally related. The flagellum transport machinery was thought to have a dedicated role in organelle biogenesis, but a recent study demonstrated that it is also required for the transport of proteins to the extracellular environment in *Yersinia enterocolitica* (Young *et al.*, 1999). Type III secretion systems have been characterised for a number of gram-negative bacteria including *Yersinia*, *Salmonella*, *Shigella* and enteropathogenic *E. coli*, in addition to unrelated plant pathogens (Galan and Collmer, 1999). Genes required for the synthesis and assembly of type III machines are typically clustered. Transfer of such a gene cluster is thought to transform otherwise nonpathogenic species into virulent organisms (Anderson and Schneewind, 1999). Indeed, the genes encoding several type III secretion systems often reside on pathogenicity islands, discrete segments of DNA that

encode virulence traits and often appear to have a foreign origin (Mescas and Strauss 1996).

Type III secretion systems have three distinguishing features: the absence in the secreted proteins of a cleavable signal peptide that is characteristic of proteins secreted via the GSP; the requirement for customised accessory proteins for many of the secreted proteins and a widespread requirement for host cell contact for full activation of the secretory pathway (Galan and Collmer, 1999). Thus, type III secretion appears to be triggered when a pathogen comes in close contact with host cells such as macrophages or epithelial cells and many proteins secreted by the Type III system are injected directly into the cytoplasm of eukaryotic cells. The effector molecules released act to alter host cell function, which facilitates the pathogen's ability to survive (China and Goffaux, 1999). The best studied model is the Yop system of Yesinia. Upon macrophage contact, yesiniae activate the type III secretion machinery to inject a set of virulence factors into the eukaryotic cell. These polypeptides, named Yersinia outer proteins (Yops) interfere with host cellular processes such as actin polymerisation and signal transduction cascades, thereby preventing phagocytosis and killing by macrophages (Lee and Schneewind, 1999). For a more detailed description of type III secretion systems, a number of recent reviews are available (Mescas and Strauss, 1996; Galan and Collmer, 1999; Anderson and Schneewind, 1999; Lee and Schneewind, 1999; Hueck, 1998).

1.9.1.4 The autotransporters

Secreted proteins that mediate their own transport out of bacteria belong to a fourth class of secretion system. Like the type II secretion system, these proteins use a sec-dependent pathway and cleavage of a classic signal sequence to translocate across the inner membrane. Their translocation across the outer membrane, however, is unique in that the protein moiety mediating export is contained within the precursor of the secreted protein itself, hence the nickname "autotransporters". The term "type IV secretion" has been proposed for these autotransporter proteins (Henderson *et al.*, 1998; Hueck, 1998), but will not be used in the context of this review, as a fifth secretion system is also referred to as Type IV (see section 1.9.1.5). The autotransporter system was first described for the IgA1 proteases *of Neisseria gonorrhoeae* but a number of

other autotransporter proteins have been recently postulated, including the VacA protein of *H. pylori* (see section 1.9.2) and Hsr of *H. mustelae* (O'Toole *et al.*, 1994).

Following translocation across the inner membrane via the general secretory pathway, passage through the outer membrane is mediated by the protein's carboxy-terminal domain and no energy coupling or accessory proteins are known to be required for this translocation process. As such, three distinct domains can be identified for nearly all autotransporter molecules: an amino-terminal leader peptide, the surface-localised "mature" protein (α or passenger domain) and a carboxyl-terminal domain (β-domain) that mediates secretion through the outer membrane. The amino-terminal peptide domain in most autotransporters contains at least some characteristics of the prototypical *sec*-dependent signal sequence described above (section 1.9.1.2) although they commonly contain unusually long signal peptides, consisting of at least 47 amino acids, the function of which has not be determined (Henderson *et al.*, 1998). Following passage across the inner membrane, the autotransporter protein exists as a periplasmic intermediate, but it is unclear how they survive the periplasmic phase without extensive degradation by periplasmic proteases. It is possible that they complex with chaperones, or exist in a protease-protected configuration or that the inner and outer membrane translocation is temporarily coupled so that the periplasmic intermediate is short-lived.

It is widely believed that the β-domain of the autotransporter protein spontaneously inserts into the outer membrane in a characteristic β-barrel configuration. A β-barrel is a complex protein structure composed of multiple amphipathic β-sheets. The amphipathic primary structure permits the establishment of a molecular pore through which the passenger domain, still attached to the β-barrel, is translocated to the cell surface. The polyprotein may then remain intact as a large protein with a membrane-bound carboxyl-terminal domain and an amino-terminal domain extending into the external milieu as is thought to be the case for Hsr of *H. mustelae* (O'Toole *et al.*, 1994). Alternatively, the protein may be cleaved by an outer membrane protease, with the passenger domain remaining in contact with the surface via noncovalent interactions or released into the external milieu, the latter being the case for the IgA1 protease. Some autotransporters are even capable of autoprocessing via their serine protease active sites (Henderson *et al.*, 1998).

1.9.1.5 Type IV secretion

Controversy surrounds the classification of this type of transport system, which is the least well characterised and comprises only three members so far. The information that follows is taken from two reviews that refer to this class of transport system as the Type IV system (Burns, 1999; Lory, 1998), despite the overwhelming bias in the literature towards the autotransporters being classified as type IV. The prototype member of the type IV transporter family is the VirB system of *Agrobacterium tumefaciens* that exports a large, single-stranded DNA, known as T-DNA, across the bacterial membranes and into plant cells, where the T-DNA integrates into the plant genome. The *virB* locus consists of 11 genes, ten of which (*virB2-virB11*) are critical for DNA transfer. A similar system of type IV secretion was recently identified in *Bordetella pertussis* and was the first type IV system shown to solely transport proteins. The *B. pertussis* secretion apparatus (Ptl system) consists of nine proteins required for the secretion of the multisubunit pertussis toxin (Lory, 1998). Additional type IV homologues were recently discovered in other pathogenic bacteria including *H. pylori* (see section 1.9.2) and *Legionella pneumophila* (Miller and Cossart, 1999).

Currently, little is known about the series of events that occur during this type of transport process. The finding that many of the VirB protein are found in both the inner and outer membrane fractions is consistent with the idea that the VirB proteins form a transport complex that spans both membranes (Burns, 1999). However, it is not yet known if transport occurs as a one-step process across both bacterial membranes simultaneously or as a two step process as with type II secretion. There is evidence to suggest that different members of the type IV secretion family differ in this regard. For instance, it has been postulated that the VirB system utilises a one-step process whereby proteins and associated DNA cross both bacterial membrane through a channel formed by the VirB proteins. The Ptl system, despite the strong homology between its proteins and that of the VirB system, is suggested to utilise a two-step process. Each of the individual subunits of the pertussis toxin that are transported by this system are synthesised with their own signal sequence, suggesting that they may cross the membrane in a sec-like system. Further investigations may elucidate the exact mechanisms behind this unusual secretion system.

1.9.2 Secretion of *H. pylori* proteins

There is accumulating evidence to suggest that surface or secreted proteins of *H. pylori* play a pivotal role in the organism's ability to colonise, persist and more importantly, promote an inflammatory response within its host (Doig and Trust, 1994). There is mounting evidence to suggest that stimulation of inflammatory cytokines, in particular IL-8, by *H. pylori* is due to the release of soluble bacterial extracellular factors or direct contact of the bacterial cell with host cells (Mai *et al.*, 1991; Huang *et al.*, 1995). Data also shows that *H. pylori* can directly injure gastric epithelial cells by the secretion of enzymes and by the elaboration of toxins. Exposure to soluble bacterial proteins leads to cell injury, which results in growth inhibition and may lead to cell death, possibly through apoptosis (Smoot, 1997).

Despite this, the mechanisms by which *H. pylori* releases or secretes its virulence factors are not yet well understood. To date, the secretion systems used to export many of the surface or secreted proteins identified in *H. pylori* remain theoretical. Sequencing of the entire *H. pylori* genome has revealed that it contains the basic mechanisms of secretion similar to those of *E. coli* and *H. influenzae*. However, some important difference were reported, notably the absence of an orthologue of the secretory chaperone, SecB (Tomb *et al.*, 1997). In addition to the SecA-dependent secretory pathway, *H. pylori* appears to contain two specialised export systems, one associated with the cag pathogenicity island (see below) and the flagellar export pathway common to gram-negative bacteria reviewed elsewhere (Porwollik *et al.*, 1999). Approximately 24% of the ORF's annotated from the *H. pylori* genome were predicted to contain a signal peptide SignalP analysis (Tomb *et al.*, 1997) and may encode for exported proteins secreted by the GSP. *H. pylori* also contains a large family of outer membrane proteins (OMP's) (Owen, 1998) that are thought to play a role in antigenic variation (Marais *et al.*, 1999), for example, the BabA outer membrane adhesin (Ilver *et al.*, 1998) and Lewis antigens (Appelmelk *et al.*, 1998). Although a clear homologue of SecB has not been found, it is to be expected that a protein with a similar function exists, as in gram-positive bacteria that lack SecB. A role for the Dnak and DnaJ proteins in the viability and protein export process of cells lacking SecB has been demonstrated (Wild *et al.*, 1992).

Several virulence factors have been described for *H. pylori*, many of which are secreted or associated with the outer membrane. These include the vacuolating cytotoxin (VacA) an 87 kDa extracellular protein. The *vac*A gene itself encodes a precursor protein of 139.6 kDa, consisting of a 33-amino acid signal sequence, the 87 kDa cytotoxin and a 50 kDa C-terminal domain (Schmitt and Haas, 1994). It is the first known protein to be actively secreted into the extracellular space and is believed to be secreted via a mechanism similar to that found in Type IV secretion systems (see section 1.9.1.4). VacA is probably specifically secreted into the extracellular space by a mechanism analogous to that used for the secretion of IgA protease from *Neisseria gonorrhoeae* (Jose *et al.*, 1995). This mechanism would involve the transport of a VacA protoxin across the inner bacterial membrane via the sec-mediated pathway, followed by transport across the outer membrane via a channel formed by the C-terminus of VacA. It is also possible that VacA activity is under the control of a secretion apparatus encoded in a second unidentified PAI (Covacci *et al.*, 1997). Detection of VacA accumulation in the periplasm has ruled out type I and III secretion pathways, while providing evidence for a two-step pathway where the N-terminal leader sequence of the 139 kDa protoxin is cleaved (Fiocca *et al.*, 1999).

The CagA gene is one of many putative virulence genes encoded by a 40 kb pathogenicity island (PAI) recently identified in *H. pylori* (Censini *et al.*, 1996) The cag PAI appears to encode a number of homologues of proteins involved in protein secretion, including flagellum assembly, proteases, translocases and permeases. Although the functions of these proteins have not been well defined, they possess features similar to components of bacterial type II, type III and most notably Type V secretion systems (Covacci *et al.*, 1997). It has been suggested that the *H. pylori cag* pathogenicity island encodes a novel secretion system for the delivery of bacterial constituents directly into host cells. These secreted products may interact directly with the epithelium to trigger a cascade of events that leads to epithelial cell damage. Alternatively, these secreted proteins could interact with immune cells that in turn liberate cytokines causing the observed effect indirectly (Mobley, 1997). It is known that the VirB4/PtlC homolog of *H. pylori*, *pic*B, plays a role in the induction of proinflammatory interleukin-8 expression in gastric epithelial cells (see section 1.7.1), since mutations in *pic*B markedly reduce the ability of the bacteria to induce IL-8 expression (Tummuru *et al.*, 1995). Evidence suggests that other components of the

PAI are involved in the induction of IL8 and research has focussed on the potential toxic factors exported by this region (Tummuru *et al.*, 1995; Censini *et al.*, 1996; Glocker *et al.*, 1998) and their role in immunopathogenesis.

In addition, various other secreted or surface-located proteins involved in motility (Suerbaum *et al.*, 1994), adhesion (Clyne and Drumm, 1996) and iron uptake (Dhaenens *et al.*, 1997) have been identified. Progress in identifying secreted or membrane proteins using standard biochemical techniques has been hampered by the presence of the high copy assemblies of cytoplasmic proteins. *H. pylori* appears unique in that three proteins, urease, HspB (a homolog of the GROEL family of heat-shock proteins), and superoxide dismutase (SOD), are associated with the outer membrane whereas they are found exclusively within the cytoplasm in other bacteria (Phadnis, 1996; Dunn *et al.*, 1997). These normally intrinsic cytoplasmic proteins become associated with the bacterial surface in a novel manner, not yet fully understood. These cytoplasmic proteins may be released by bacterial autolysis and become adsorbed to the surface of intact bacteria due to the unique characteristics of the outer membrane (Phadnis *et al.*, 1996; Cao *et al.*, 1998). Such "altruistic lysis" appears to be essential in our understanding of the pathogenesis of *H. pylori* and may explain how vaccines against a cytoplasmic protein such as urease can be effective against *Helicobacter* sp in animal models. Indeed, it was recently demonstrated that the release of urease by altruistic lysis is essential for the survival of *H. pylori* in an acid environment (Krishnamurthy *et al.*, 1998). It may also explain how the integral membrane proteins of *H. pylori* can evade immune detection and persist in spite of a humoral and cellular immune response and how this noninvasive bacterium can present virulence factors and immunogens to the immune system (Dunn *et al.*, 1997).

One method of ascertaining the location of a protein within a bacterium is to determine its localisation index. The localisation index is an expression of secretion efficiency. Cytoplasmic proteins should, by definition, have a localisation index of zero. Differentiation between antigens that are actively secreted from the cell and intracellular antigens released during the autolytic process during culture has met with considerable difficulty in the past, although these two groups are functionally quite distinct. The use of cytoplasmic marker proteins with simultaneous quantification of these and other individual proteins has been essential in defining these groups of proteins in other

bacteria (Harboe *et al.*, 1992). Using a similar technique, some *H. pylori* proteins have been shown to become associated with the outer membrane by a novel programmed release mechanism (Vanet and Labigne, 1998) rather than autolysis.

1.9.2.1 Outer membrane vesicles and *H. pylori*

A unique feature of gram-negative bacteria is the discharge from their surface of outer membrane vesicles (OMVs) during bacterial growth. As these vesicles are being extruded from the surface, they entrap some of the underlying periplasm so that they are actually small particles of gram-negative cell wall. They possess OMPs, LPS, phospholipids, and periplasmic constituents and are released by virtually all gram-negative bacteria (reviewed in Beveridge, 1999).

The pathogenicity of a variety of gram-negative bacterial pathogens relies, at least in part, on their ability to secrete a number of virulence factors into the environment surrounding their targeted tissue. OMVs could provide an alternative route for the delivery of virulence factors. *H. pylori* releases OMVs during growth *in vitro* and *in vivo* and these vesicles contain VacA (Fiocca *et al.*, 1999). It has been observed that *H. pylori* vesicles bind to epithelial cells *in vitro* as well as to the gastric mucosal surface *in vivo* (Fiocca *et al.*, 1999) and may represent a vehicle whereby *H. pylori* virulence factors are delivered to the gastric mucosa. Since *H. pylori* rarely enter intact epithelial cells, it has remained poorly understood how infection with *H. pylori* results in a vigorous local and systemic immune response. The data indicates that *H. pylori* vesicles frequently enter the endocytic pathway of epithelial cells. Antigens that are released from these vesicles may traverse the gastric epithelial monolayer and ultimately reach the lamina propria via a process involving transcytosis. (Fiocca *et al.*, 1999).

1.10 Aims of the Thesis

The objective of the present study was to identify, using alkaline phosphatase (AP) gene fusion technology, novel *H. pylori* sequences directing protein export and to evaluate the antigenicity of the fusion proteins using using blood samples taken from *H. pylori* infected patients. In order to identify exported proteins that are potentially immunogenic during *H. pylori* infection, two approaches were employed to screen the *H. pylori* AP fusion proteins generated, (1) Western blot screening of the fusion proteins using patient sera, (2) lymphocyte proliferation assays using peripheral blood mononuclear cells from *H. pylori* infected patients. It was expected that this strategy would generate a number of immunogenic proteins which could then be further characterised and tested for their ability to induce protective immunity in a mouse model of infection. The specific aims of the thesis were to:

1. Construct a *Helicobacter pylori* genomic library in the shuttle plasmid vector, pJEM11, and identify *H. pylori* gene sequences encoding exported proteins.

2. Screen the *H. pylori* alkaline phosphatase fusion proteins generated from the pJEM11 library with patient sera and test for their ability to induce proliferation of peripheral blood lymphocytes.

3. Construct an expression library of *H. pylori* genomic DNA in the Lambda Zap vector to obtain the full gene sequence of any exported proteins identified from the pJEM11 library (this was done prior to the release of the *H. pylori* genome sequence).

4. Clone and express selected genes, identified by the immunological screening, to obtain purified recombinant protein for further immunological characterisation.

5. Test recombinant proteins in a mouse model of *H. pylori* infection for their ability to induce protective immunity.

6. Identify the gene encoding an antigenic outer membrane vesicle component by screening the *H. pylori* expression library with a monoclonal antibody raised to this antigen (This formed part of a collaborative project with the Christchurch School of Medicine, New Zealand).

Chapter 2 General Materials and Methods

2.1 Bacterial strains and plasmids

2.1.1 Bacterial strains

The bacterial strains used in this study are detailed in Table 2.1:

Table 2.1: Bacterial strains used in this study

Strain	Genotype/Phenotype	Source/Reference
E. coli DH10B	*F'mcrAΔ(mrr-hsdRMS-mcrBC)f80 φlacZΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ(ara,leu)7697 galU galK* λ^- *rpsL nupG*	Sambrook *et al.* (1989)
E. coli XLI-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F' proAB* $lacI^qZ\Delta M15$ *Tn10* (tet^r)*]*	Sambrook *et al.* (1989)
E. coli BL21(DE3)	*hsdS gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)*	Sambrook *et al.* (1989)
H. pylori 17874	*H. pylori* type strain, Cag A^+/Vac A^+ (Type I), identical to NCTC 11637	Culture collection, University of Gothenburg, Sweden.
H. pylori NZCH1	Cag A+ Vac A+	Otago University, NZ
H. pylori MU005	Clinical isolate, Auckland, Cag^+/$VacA^+$ (Type I)	Campbell *et al.* (1997)
H. pylori MU007	Clinical isolate, Auckland, Cag^+/$VacA^+$ (Type I)	Campbell *et al.* (1997)
H. pylori MU008	Clinical isolate, Auckland, $CagA^-$/$VacA^-$ (Type II)	Campbell *et al.* (1997)
H. pylori MU015	Clinical isolate, Auckland, $CagA^-$/$VacA^-$ (Type II)	Campbell *et al.* (1997)
H. pylori MU016	Clinical isolate, Auckland, Cag^+/$VacA^+$ (Type I)	Campbell *et al.* (1997)
H. pylori MU038	Clinical isolate, Auckland, $CagA^-$/$VacA^-$ (Type II)	Campbell *et al.* (1997)
H. pylori MU064	Clinical isolate, Auckland, Cag^+/$VacA^+$ (Type I)	Campbell *et al.* (1997)
H. pylori MU091	Clinical isolate, Auckland, $CagA^-$/$VacA^-$ (Type II)	Campbell *et al.* (1997)
H. felis	Cat clinical isolate	P.W.O'Toole,
H. mustelae 4298	Laboratory passaged strain	P.W.O'Toole*

* originally obtained from J.G.Fox, Massacusetts Institute of Technology, Ma, USA.

All *E. coli* strains were obtained from commercial suppliers. All *Helicobacter* species and *H. pylori* isolates were kindly supplied by P.W.O'Toole, Massey University, NZ, with the exception of clinical isolate NZCH1, which was originally obtained from Professor V.S. Chadwick, Otago University, NZ.

2.1.2 Plasmids and phagemids

The plasmids used in this study and their relevant characteristics are detailed in Table 2.2. The mycobacterial shuttle vector, pJEM11, was kindly supplied by Brigitte Gicquel, Unite de Genetique Mycobacterienne, Pasteur Institute, Paris, France. All other *E. coli* expression vectors and phagemids were obtained from commercial sources.

Table 2.2: Plasmids used in this study

Plasmid	Description	Source / Reference
pBK-CMV	Phagemid vector (P_{lac},$f1_{ori}$,$ColE1_{ori}$,$SV40_{ori}$,Kan^r)	Stratagene
pJEM11	*E. coli* - mycobacterial shuttle vector containing a truncated *phoA* gene, Kan^r	Lim *et al.*, 1995
pJHp1-294	pJEM11 containing 0.2-2 kb fragments from *Helicobacter pylori* NZCH1	This study
pJHp20	pJEM11 containing a 1.1kb *H.pylori* fragment containing the ORF HP1085	This study
pXa1	Expression vector (p_{lac}; $f1_{ori}$:)	Boehringer Mannheim
PXa-1085	pXa1 vector containing the 20/1-20/2 PCR amplification product	This work
PPROEX HTb	Expession vector (Ptrc, $lacI^q$, $f1_{ori}$,, Amp^r, pBR322 ori)	Life Technologies
pPRO-1456	pPROEX HTb containing the 1456/B-1456/E PCR product	This study
pPRO-1457	PPROEX HTb containing the 1457/B-1567/E PCR product	This study
PGEX-6P-3	Expression vector (Ptac, $lacI^q$, $f1_{ori}$,, Amp^r, pBR322 ori)	Pharmacia Biotech
PGEX-1456	PGEX-6P-3 containing the 1456/B-1456/E PCR product	This study
PGEX-1457	PGEX-6P-3 containing the 1457/B-1457/E PCR product	This study

2.2 Bacterial growth conditions

E. coli strains were routinely grown in Luria Bertani (LB) broth or on LB plates (1% (w/v) tryptone, 0.5% (w/v) yeast extract (Difco), 0.5% (w/v) NaCl, pH 7.0)) at 37°C under aerobic conditions (Miller, 1972). All liquid cultures were aerated at approximately 200 rpm. *Helicobacter* cultures were routinely grown on Columbia agar base (Oxoid) supplemented with 5% sheep blood. Plates were incubated under microaerophillic conditions consisting of 5% oxygen, 10% carbon dioxide and 85% nitrogen. Plates were incubated for approximately 4 to 5 days at 35°C. Recombinant *E.*

coli were grown overnight at 37°C in 5 ml of the appropriate media containing the appropriate antibiotic (Sigma, St. Louis). For medium term storage, plates were kept at 4°C. For long term storage of bacteria, liquid cultures were grown overnight, mixed with 15% (v/v) glycerol and stored at –70°C.

Table 2.3: Media supplements and antibiotics

Supplement	Stock concentration (mg/ml)	Final concentration (μg/ml)
Ampicillin	100	100
Kanamycin	20	30
BCIP	40	25
IPTG*	240	25
X-gal*	20	20

* for plating the recombinant libraries in *E. coli* DH10B and *E. coli* XLI blue.

2.3 DNA extractions

2.3.1 Isolation of plasmid DNA from *E. coli*

Plasmids were isolated from *E. coli* by the alkaline lysis mini-prep method as previously described (Ish-Horowicz and Burke, 1981). Briefly, *E. coli* was grown overnight under standard conditions and the bacterial cells harvested by centrifugation. The cells were resuspended in buffer I (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA) followed by the addition of lysis buffer II (0.2 M NaOH, 1% SDS) and incubated on ice. Following the addition of ice-cold buffer III (5 M K- acetate, glacial acetic acid), the precipitated protein, SDS and chromosomal DNA were removed by centrifugation. The supernatant containing the plasmid DNA was then extracted twice with phenol-chloroform followed by ethanol precipitation. Alternatively, plasmid DNA was prepared using either the QIAprep-spin Plasmid Miniprep Kit (QIAGEN, Hildon, Germany) or BRESAspin Plasmid Mini Kit (Bresatec, Australia).

2.3.2 Isolation of genomic DNA from *Helicobacter* species

The *Helicobacter* species and strains used in this study are listed in Table 2.1.1 For DNA extraction, the organisms were grown under standard conditions and the cells harvested from two media plates into 1.5 ml TE buffer. Cells were pelleted by centrifugation at 2,000 rpm for 10 mins, washed once with TE buffer and resuspended in 800 µl of TE buffer. To this suspension, 100 µl of 10 mg/ml lysozyme was added and the tube incubated in a 37°C waterbath for 30 mins. After the addition of 100 µl of 10% SDS, the tube was incubated for a further 30 mins at 37°C. Incubation was increased to 56°C for 1 hour after the addition of 5 µl of 10 mg/ml Proteinase K. The DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) to remove contaminating protein and ethanol precipitated at -75°C for 1 hour. The DNA was then resuspended in 100 µl of distilled water. DNA concentrations were measured using a GeneQuant (Pharmacia) at 260 nm.

2.4 DNA manipulations

2.4.1 Ethanol precipitation of DNA

DNA was routinely precipitated by the addition of 2.5 volumes of 95% (v/v) ethanol and 0.1 volume of 3 M Na-acetate (pH 5.2). The solution was then put at –20°C for at least 30 minutes. The DNA was pelleted by centrifugation at 14,000 rpm for 30 mins, washed with 70% ethanol and the pellet vacuum dried in a Speed Vac (SC100, Savant). DNA was resuspended in either TE buffer or distilled water.

2.4.2 Electrophoresis

Agarose gels were prepared in 1x TBE or TAE buffer, usually at a concentration of 1% agarose (Life Technologies). The agarose was dissolved by heating the solution in a microwave and following cooling, ethidium bromide was added to a final concentration of 10 µg/ml. Gels were poured and electrophoresis was performed using BioRad equipment according to the manufacturer's instructions. Samples were loaded with 0.2 volumes of loading buffer (0.25% (w/v) bromophenol blue, 30% (v/v) glycerol, 0.25% (w/v) xylene cyanol) and run alongside the appropriate DNA marker. TBE or TAE buffer (1x) were used as the running buffer and the DNA fragments were separated by electrophoresis with a limiting current of 80 mA for small gels, or 100 mA for large

gels. The molecular size of the DNA fragments was estimated by comparison with one of the following DNA markers: ϕX174 RF DNA / *Hae* III fragments, Lambda DNA / *Hin*d III fragments and 1 kb ladder (GIBCO BRL, Life Technologies). The gels were exposed to UV light and photographed using a Polaroid camera with Polaroid 667 film.

2.4.3 Cloning procedures

2.4.3.1 Digestion of DNA with restriction enzymes

A typical restriction enzyme digest was performed in a final volume of 50 μl containing 0.2 – 1 μg of target DNA. Enzymes were purchased from Boehringer Mannheim unless otherwise stated. The appropriate enzyme buffer was added to a final of 1x along with 1 –2 units of the enzyme. One unit of enzyme was defined as the amount required to digest 1 μg of DNA to completion in one hour in the recommended buffer at the recommended temperature. Reactions were generally performed at 37°C for one to two hours and the reaction stopped by the addition of 10 mM EDTA (pH 8.0).

2.4.3.2 Ligation of DNA into plasmid vectors

In general, ligations were performed using fragments with noncomplementary protuding termini generated by digestion two different restriction enzymes. Approximately 100 ng of vector DNA was added to an equimolar amount of insert DNA in a 20 μl volume. Ligase buffer (1x) and T4 DNA Ligase (1 unit) were added to the DNA and the reaction incubated at 10°C for 5 min – 1 hour. Since ligation of blunt-ended fragments is comparatively inefficient, these concentrations of both the DNA termini and DNA ligase were increased and the reaction carried out at 25°C overnight. DNA Polymerase I (Klenow Fragment) was used to fill in recessed 3' termini created by digestion of DNA with restriction enzymes to achieve blunt ends according to standard procedures (Sambrook *et al.*, 1989). Where fragments of DNA carrying identical termini were to be cloned into linearised plasmid DNA bearing compatable ends, the vector was treated by standard procedures using calf intestinal alkaline phosphatase (CIAP) to remove the 5' phosphate group and prevent self-ligation using standard procedures (Sambrook *et al.*, 1989).

2.5 Transformations

2.5.1 Preparation of competent cells

For preparation of competent cells, *E. coli* cultures were grown to log phase and repeatedly exposed to calcium, which renders the cells amenable to uptake of DNA, as follows. A single colony of the *E. coli* was innoculated into 5 ml of LB media and grown overnight at 37°C with moderate shaking (250 rpm). Four millilitres of the culture was sub-innoculated into 400 ml of media in a sterile flask and grown at 37°C with shaking to an OD_{600} of 0.3 - 0.75. The cells were then centrifuged for 10 mins at 5000 rpm at 4°C. The supernatant was poured off and the cells gently resuspended in 4 ml ice-cold 0.1 M $CaCl_2$ solution. The cells were centrifuged a further 10 mins at 5000 rpm at 4°C and the pellet gently resuspended in another 4 ml 0.1 M $CaCl_2$ and incubated on ice for approximately 15 minutes. Following a final centrifugation of 10 mins at 5000 rpm at 4°C, the cells were resuspended in 2 ml 0.1 M $CaCl_2$, 20% glycerol, aliquoted and stored at -70°C. For the preparation of electrocompetent *E. coli* cells, the above procedure was adjusted to include ice-cold distilled water in place of $CaCl_2$.

2.5.2 Transformation of *E. coli*

Competent *E. coli* cells were transformed with either 10 ng of purified plasmid or approximately 100 ng of the ligation mix by heat-shock at 42°C for 90 secs using standard procedures (Sambrook *et al.*, 1989). *E. coli* ElectroMAX DH10B cells (GIBCO BRL, Life Technologies, Inc.) and *E. coli* XL1-blue cells (Stratagene) were transformed with purified plasmid DNA or the ligation mix by electroporation according to the manufacturer's instructions. All ligation mixes were dialysed for 10 mins using 0.025 μM pore filter discs (Millipore Corporation, Bedford, MA) prior to electroporation. The following electroporation conditions were used with the Bio-Rad Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA): cells with a 0.2 cm electrode gap, 2.5 kV, 25 μF capacitance, and 200 ohms resistance. Transformed *E. coli* were incubated in 1 ml LB broth at 37°C for approximately 1 hour prior to plating on LB plates containing the appropriate antibiotic.

2.6 Polymerase chain reaction

2.6.1 Primer design

Oligonucleotide primers were generally designed based on published sequences. Several factors were taken into consideration when designing primers for PCR or sequencing reactions. Except in the case where restriction enzyme sites were to be incorporated, all primers were 100% specific for the template, with no sequence mismatches. The Amplify version 1.2 software program was used to check for alternative hybridisation sites present in the template and the possibility of primer-dimer formation. In addition, primers were assessed for the presence of secondary structures. All primers were designed to be of an adequate length to give good specificity and melting temperatures, usually between 20 and 30 base pairs. For primers shorter than 20 base pairs, the melting temperature (Tm) was calculated according to the formula Tm = 2°C[A+T] + 4°C[G+C]. An average G+C content around 50% was maintained where possible. Oligonucleotide primers were designed with the aid of the GeneWorks™ software. Primers were synthesised by Life Technologies and resuspended in distilled water to a final concentration of 100 µM. To determine the volume of water (mls) in which to dissolve the oligonucleotide, the following formula was used:

$$\frac{\text{Total OD (Absorbance at 260nm)}}{[\text{ (EM) x Molar Concentration Required }]}$$

where EM is the molar extinction coefficent.

2.6.2 PCR conditions

Standard PCR reactions were carried out using ***Taq*** DNA polymerase unless otherwise stated according to the manufacturer's instructions, in the presence of 1.5 mM $MgCl_2$ in a 50 µl volume. The following cycling parameters were typical for a standard PCR: 95°C for 2 min, followed by 30 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec, with an extended elongation step (72°C for 5 min) for the final cycle. Amplification reactions were carried out in a Perkin Elmer 9600 PCR machine using 0.2 ml thin-walled PCR tubes (Life Technologies). The PCR amplification products were analysed by electrophoresis on agarose gelS (see 2.4.2). PCR products were purified directly using a PCR purification kit (Boehringer Mannheim) or alternatively, if

more than one band was observed, were purified from the agarose gel using a gel purification kit (Qiagen, Aus). Procedures for DNA template preparation, preparation of the PCR mix and PCR cycling were all carried out in designated separate laboratories to reduce the risk of contamination.

2.7 DNA sequencing

2.7.1 Manual sequencing

Manual sequencing was performed using Life Technologies Sequencing gel electrophoresis apparatus Model S2 according to the manufacturer's instructions. Standard 6% polyacrylamide/urea sequencing gels were prepared as follows. Fifteen millilitres of a 40% acrylamide solution (BioRad) was added to 1x TBE along with 50 g urea in a final volume of 100 ml. The solution was degassed under vacuum before the polymerising agents (1% ammonium sulphate, 15 µl TEMED) were added. The gel was poured at a 45° angle to minimise air bubbles. Following polymerisation, the gel sandwich was secured to the apparatus and pre-electrophoresed for 15 mins prior to loading samples.

Plasmid DNA templates to be sequenced were prepared using the Qiagen plasmid kit, followed by ethanol precipitation and PCR products purifed using a Breatec DNA purification Kit (Bresatec, Aus). For determination of the complete sequence, four reactions, one for each nucleotide (dG,dA,dT,dC) were carried out. Each reaction contained the DNA template (1-100 fmol), the sequencing primer (20 µM) with [α^{33}P] for direct label incorporation, Taq DNA polymerase, each deoxynucleoside triphosphate (dGTP, dATP, dTTP, dCTP) and one of the four dideoxynucleoside triphosphates (ddNTPs). The cycling protocol consisted of a denaturation step at 95°C, annealing step of 55 – 70°C depending on the sequencing primer, and an extension/termination step at 72°C. The extension step is terminated by the incorporation of a ddNTP. Three microlitres of the labelled products were electrophoresed at 1,500-1,900 volts/30-45 mA/60 Watts for 3-6 hours. The gel was then transferred to Whatman paper, dried and exposed to X-ray film followed by autoradiography to visualise the products.

2.7.2 Automated sequencing

Automated sequencing was carried out by Massey University DNA Analysis Service (MUSeq). This service provides automated DNA sequencing on an ABI Prism 377 DNA Sequencer (Perkin Elmer). The reactions were performed using a Dye Terminator Cycle Sequencing Ready Reaction Kit. This kit uses dye labelled dideoxynucleotides in which a specific fluorescent dye is covalently attached to each base. Products can then be visualised as fluorescent colour coded bands that are collected and interpreted by computer software.

2.7.3 Sequence analyses

DNA and protein databases were searched to identify similarities between the insert DNA and previously identified sequences. Databases used for sequence similarity searches included the National Centre for Biotechnology Information (NCBI) BLAST server (http://www.ncbi.nlm.nih.gov/index.html) (Altschul *et al.*, 1990) and European Bioinformatics Institute FASTA server (http://www.ebi.ac.uk/ebi_home.html) . The BLAST + BEAUTY algorithm (Worley *et al.*, 1995) was also used to search databases for sequence similarities and structural motifs. Following the release of two entire *H.pylori* genome sequences (Tomb *et al.*, 1997, Alm *et al.*, 1999), the *H.pylori* genome databases (http://www.tigr.org/tdb/mdb/hpdb/hpdb.html and http://www.astra-boston.com/hpylori/). were searched to identify sequence similarities with the annotated *H. pylori* open reading frames (ORF's). The GeneWorks programme (release 2.45, IntelliGenetics, Inc. California) was used for sequence analyses. In addition, the ExPASy Molecular Biology Server (http://expasy.hcuge.ch), SignalP World Wide Web Prediction Server (http://www.cbs.dtu.dk/servers/SignalP/), PSORT II (http://psort.nibb.ac.jp/) (Nielsen *et al.*, 1997) and Promoter Prediction (http://www-hgc.lbl.gov/projects/promoter.html) (Reese *et al.*, 1996) were utilised in the analysis of *H. pylori* export proteins.

2.8 DNA hybridisations

2.8.1 Isolation and labelling of DNA probe

2.8.1.1 Radioactive probes

DNA fragments to be used as probes in Southern blot experiments were routinely purified from agarose gels. Following electrophoresis, the DNA fragments were eluted from a 1% low melting point agarose gel using a GELase kit (Epicentre Technologies Corporation, Madison, Wisconsin). DNA fragments were labelled with [α-^{32}P]-dCTP using the RTS RadPrime DNA labelling system (GIBCO BRL, Life Technologies). This system is based upon the random primer labelling method and produces probes with specific activities of greater than 10^9 cpm/µg. The nick-translated DNA fragments were separated from unincorporated radiolabelled nucleotides using a NICK column (Pharmacia Biotech, Uppsala, Sweden). Following equilibration of the column with buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA), 50 µl of the sample was added, washed with 400 µl of buffer, and eluted with a further 400 µl of buffer solution. The fraction containing the radiolabelled probe was either used immediately or stored at –20°C until required.

2.8.1.2 Digoxigenin probes

As an alternative to radioactive labeling, the DIG nonradioactive nucleic acid labeling and detection system was used in Southern blotting (Boehringer Mannheim). DNA fragments were routinely extracted with phenol:chloroform prior to labeling. DNA was labeled with didoxigenin using the standard random primed DNA reaction. In this reaction, one digoxigenin molecule is incorporated in every 20 – 25 nucleotides and yields a minimum of 250 ng of digoxigenin-labeled probe from 1 µg of DNA template in 1 hour. Briefly, the DNA to be labeled was denatured by boiling for 10 mins followed by the addition of 4 µl of DIG-High Prime (Boehringer Mannheim). The reaction was incubated at 37°C overnight and stopped by the addtion of EDTA. The yield of DIG-labeled probe was quantitated by the spot test in which a side by side comparison of the DIG-labeled probe with a DIG-labeled control is carried out. A serial dilution of the labeled probe and control sample were spotted onto the membrane

and colorimetrically detected according to the manufacturer's instructions (Boehringer Mannheim).

2.8.2 Southern blot analysis

2.8.2.1 Radioactive Southern blotting

DNA fragments were size-fractionated on 1.0% agarose gels by electrophoresis, incubated in denaturation (*) solution followed by neutralisation solution (#) for 15 mins. The DNA was blotted onto nylon membrane (Hybond-N, Amersham International, U.K.) by capillary transfer using standard procedures (Sambrook *et al.*, 1989). The DNA was fixed to the nylon membranes by exposure to UV irradiation for 3.5 mins. Hybridisation was carried out using Rapid-hyb buffer (Amersham) as recommended by the manufacturer. Unless stated otherwise, the membranes were incubated at 65^oC for 2 hrs in the rapid hybridisation buffer containing 50-100 µl of [$\alpha^{32}P$]-labelled probe. Washes were carried out in 2 x SSC (Appendix x), 0.1% SDS at RT for 10 min, followed by 1 x SSC, 0.1% SDS at 65^oC for 2 x 15 min. To re-use Southern blots, the membranes were incubated at 45^oC for 30 min in 0.4 M NaOH and then transferred to a solution containing 0.1 x SSC, 0.1% SDS, 0.2 M Tris-HCl pH 7.5 for 30 min. Autoradiographs were prepared by exposure to X-ray film (Kodak X-Omat AR) at -75^oC for at least 16 hrs in the presence of a single intensifying screen.

2.8.2.2 DIG Southern blotting

Membranes were prepared as above and prehybridised in DIG Easy Hyb buffer (Boehringer Mannheim) at 42^oC for 2 hours. Following prehybridisation, the blot was hybridised with 25 ng probe / ml hybridisation solution overnight at 42^oC followed by washing twice in 0.5xSSC, 0.1% SDS at 68^oC. The membranes were then incubated in blocking solution for 30 - 60 mins and developed using the anti-DIG antibody conjugated to alkaline phosphatase dilutied 1:10,000 according to the manufacturer's instructions. The blots were developed by either colorimetric or chemiluminescent detection using NBT/BCIP or CSPD substrate respectively. After 5 mins incubation in CSPD substrate, the membrane was exposed to X-ray film and developed for 15 – 20 mins.

* Denaturation solution: 0.5 M NaOH, 1.5 M NaCl
# Neutralisation solution: 1 M Tris-HCl, 1.5 M NaCl, pH 7.5

2.9 Protein preparations

2.9.1 Preparation of *E.coli* lysate

Recombinant *E. coli* clones were grown in LB culture medium supplemented with 30 μg/ml kanamycin at 37°C on an orbital shaker for approximately 16 hrs. Cultures were inoculated 1:100 into 25 ml of fresh medium and grown to mid-log phase. The cells were washed twice with 25 mM Tris-HCl, 2 mM $MgCl_2$ (pH 7.0) buffer by centrifugation at 3000 x g for 10 min at 4°C, and resuspended in 5.0 ml of wash buffer. Cells were sonicated on ice for 3 x 1 min at 30 sec intervals using an XL-2020 Sonicator (Heat Systems Inc., Farmingdale, NY) with microtip on setting 3.5. The cells were incubated on ice for 10 mins in the presence of approximately 200 units of DNase I (BRL, Life Technologies, Auckland) after which was added 1 mM PMSF and 2.5 mM EDTA (pH 8). Insoluble cellular debris was removed by centrifugation at 14,000 rpm for 10 min at 4°C in a microfuge and the supernatants collected. A non-recombinant E. coli DH10B lysate control sample was prepared according to the above protocol from a 25 ml culture in the absence of kanamycin.

2.9.2 Preparation of a *H. pylori* lysate

H. pylori was grown under the appropriate conditions on blood agar plates and lysate samples prepared from cells scraped from the solid media. Colonies were scraped from plates in a 1.5 ml volume of TE (pH 8) using a glass rod. The cells were pelleted in a microfuge and the supernatants (extracellular wash samples) removed. The *H. pylori* cells were washed twice with TE (pH 8) and resuspended in 5 ml of TE. Cell lysates were then prepared by sonication as described in section 2.9.1.

2.10 Protein analysis

2.10.1 Estimation of protein concentration

Protein concentrations were estimated in duplicate using the BCA Protein Estimation Assay (Pierce, Rockford) according to the manufacturer's instructions. The BCA assay is based upon a colour change resulting from a complex formed between bicinchoninic acid and Cu^{+1}, which is produced when Cu^{+2} is reduced by protein in an alkaline environment. Briefly, protein samples were diluted in 1 ml dH_2O, to which was added 1

ml of premixed colour reagent (Pierce) and the sample incubated at 37°C for 30 mins. A set of bovine serum albumin (BSA) protein standards was also prepared in the same manner. Protein was estimated by reading the absorbances at 562 nm and comparing to a standard curve generated by the known standards. Purified recombinant protein was determined by reading the abosrbance at 280 nm and the amount calculated according to the individual molar coefficient.

2.10.2 Polyacrylamide gel electrophoresis (PAGE)

Protein separation was carried out on SDS polyacrylamide gels (SDS-PAGE) using BioRad mini-gel electrophoresis apparatus according to the manufacturer's instructions. The resolving PAGE gels were prepared with 1.5 M Tris pH 8.8 with a 5% stacking gel prepared with 0.5 M Tris pH 6.8. The electrophoresis buffer consisted of 192 mM glycine, 25 mM Tris and 0.03% SDS (Laemmli, 1970). Kaleidoscope prestained proteins were used as size standards (BioRad Laboratories, Auckland). Following electrophoresis, proteins were visualised by staining with Coomassie Brilliant Blue R-250. For Coomassie staining, the gels was first incubated in fixing solution (40% methanol, 10% acetic acid), followed by staining with 0.25% (w/v) coomassie powder in fixing solution. Once the protein bands were visualised, the gel was destained in 10% ethanol, 5% acetic acid.

2.10.3 Western blotting

For Western blotting experiments, protein samples were run on an SDS-polyacrylamide gel and transferred onto PVDF membrane (NEN™ Life Sciences Products, USA) using a mini-Transblot apparatus (Bio-Rad Laboratories, Auckland). Firstly, the SDS-PAGE gel was equilibrated by soaking in blotting buffer (48 mM Tris, 39 mM Glycine, SDS, 20% methanol, pH 9.2) twice for 10 minutes with shaking. Ten sheets of filter paper cut to the size of gel were also soaked in blotting buffer twice for 10 minutes. PVDF membrane was pre-wetted in methanol according to the manufacturer's instructions prior to equilibration in blotting buffer. Five sheets of the presoaked filter paper was placed on the anode, followed by the membrane and finally the PAGE gel, ensuring there were no air bubbles. The remaining filter paper was used to cover the gel and the cathode placed on top. Membranes were blotted for 30 –60 mins at 20V (using BioRad power pack Model 200/2.0). Kaleidoscope molecular weight standards (BioRad) were

run on gels alongside protein samples. Following blotting, the membranes were incubated in a 2% milk powder solution in PBS / 0.1% tween-20 (PBS-T) for 2 hrs at RT to block sites without bound protein. Membranes were incubated with the appropriate primary antibody diluted in PBS-T at RT for 1-2 hours. Following washing with PBS-T, membranes were incubated with the corresponding secondary antibody conjugated to alkaline phosphatase conjugate at RT for 1 hr. Following incubation, the membranes were washed twice with PBS-T and once with substrate buffer (100 mM Tris / 100 mM NaCl / 10 mM $MgCl_2$). Protein bands detected by the antibody were visualised by the addition of 50 μg/ml XP and 0.01% nitro-blue tetrazolium as substrates (Sigma, St. Louis).

Alternatively, to increase the sensitivity, Western blots were developed using a Chemiluminescence Western blotting kit (Boehringer Mannheim). Membranes were developed as for colorimetric detection with the following modifications: Tris buffered saline pH 7.5 (TBS) was routinely used as the washing buffer and membranes were incubated in 1% blocking solution. Following incubation with the primary antibody, blots were developed using a POD-labeled secondary antibody at 40 mU/ml. Protein bands were detected by a 60 sec incubation in detection solution (Boehringer Mannheim) followed by short exposures to X-ray film according to the manufacturer's instructions.

2.11 Patient serum

2.11.1 Patient details

Sera was collected from thirty-five patients at the Wakefield Gastroenterology Centre, Wellington. Of the total thirty-five patients, twenty –three were determined to be infected with *H. pylori*. Infection was diagnosed by either CLO test (C), urease breath test (UBT) and/or by testing for anti-*H. pylori* antibodies in serum (Quikview Kit). Control sera was obtained from twelve asymptomatic volunteers who were determined to be H. pylori negative by Quikview and CLOtest. For a description of patient details see Table 2.4. Blood samples (5-10 ml) were collected into a Vacu-tainer (Becton-Dickinson) and left to clot for approximately 2 hrs after which the serum was collected by centrifugation at 3,000 rpm for 15 - 20 mins. This study was approved by the

Wellington Ethics Committee, Central Regional Health Authority, New Zealand. Patient anonymity and confidentiality were maintained.

2.11.2 Removal of *E. coli* antibodies

Crude sera and ascites fluid often contains IgG components that bind to *E. coli* proteins and may cause cross-reactivity. Adsorbing the sera with *E. coli* protein can inhibit or remove the anti - *E. coli* IgG's, and nonspecific binding may be decreased. *E. coli* antibodies were removed from sera samples using reverse affinity chromatography. The method immobilises *E. coli* proteins onto an agarose column to which the primary antibody preparation (sera) is applied directly for the removal of *E. coli* reactive antibodies. The column was packed with *E. coli* lysate slurry according to the manufacturer's instructions (Pierce, Rockford, Illinois, USA). The column was then equilibrated with 10 ml of TBS pH 7.6, and 100 µl of serum in 1 ml TBS applied to the column. Fractions were eluted by adding 1 ml of TBS and after reading the absorbance at A_{280}, fractions containing the highest amount of protein were pooled and diluted 1:200 for Western blotting.

Table 2.4: Patient data for sera used in this study

Serum Number	Age	Gender	HP Status
PS001	60	M	N/C
PS003	32	M	P/U
PS005	45	M	N/C
PS006	72	M	P/U
PS007	38	F	N/C
PS008	47	F	P/U
PS009	47	F	P/U
PS010	44	F	P/C
PS014	33	F	P/C,U
PS017	74	M	P/C
PS018	49	F	P/C
PS019	42	M	P/Q
PS023	28	F	N/C
PS025	64	M	P/C
PS027	69	M	P/C
PS028	49	M	P/C
PS033	66	M	P/Q
PS036	57	F	N/C,Q
PS038	57	F	N/C,Q
PS039	67	M	P/C,Q
PS040	29	F	P/C
PS045	63	M	P/C
PS046	68	M	P/C
PS048	39	M	P/C
PS062	63	M	P/C,Q
PS064	49	M	P/Q
PS066	42	M	P/Q
PS068	55	F	N/Q
PS069	33	F	N/Q
PS070	55	M	P/Q
PS071	48	F	N/Q
PS072	25	F	N/Q
PS073	47	F	N/Q
PS074	32	M	N/Q
PS075	31	F	N/Q
PS076	40	M	P/Q

HP Status: N: *H. pylori* negative, P: *H. pylori* positive
C: Clotest (biopsy urease test); U: urease test using C14; Q: QuikView (fingerprick whole blood test).

Chapter 3 Development of an *Helicobacter pylori* Expression Library for the Identification of *H. pylori* Genes Encoding Immunogenic Proteins

3.1 Abstract

Characterisation of the humoral immune response to *Helicobacter pylori* infection in humans has facilitated the investigation of the host response to bacterial virulence factors and the development of serological tests that are both sensitive and specific in the diagnosis of the infection. This study describes a preliminary investigation into the recognition of *H. pylori* antigens by sera taken from *H. pylori*-infected and non-infected patients. A clinical isolate of *H. pylori* was used as the source of antigen. This strain was demonstrated to be a Type I *H. pylori* strain on the basis of production of both the vacuolating cytotoxin and cytotoxin-associated protein. Differential screening of *H. pylori* total cell protein against sera from *H. pylori* infected and non-infected patients showed that a number of *H. pylori* proteins were recognised by antibodies present only in the infected patient sera. In other experiments, the *H. pylori* isolate was used as the source of DNA for construction of a *H. pylori* genomic expression library in the bacteriophage, Lambda. Screening of the library with gene probes derived from previously characterised *H. pylori* genes identified the urease B subunit and flagellin A subunit genes and immunoscreening of the library with a monoclonal antibody enabled the identification of a gene encoding an outer membrane vesicle antigen of *H. pylori*.

3.2 Introduction

Since its discovery over a decade ago in 1982, *Helicobacter pylori* has been the subject of extensive research aimed at improving diagnosis and control of the infection, and the identification of *H. pylori* antigens remains critical to this challenge. Initial studies investigating the protein profiles of *H. pylori* strains using SDS-PAGE, demonstrated that there was a high degree of conservation between strains (Perez-Perez *et al.*, 1987). This is in contrast to the high degree of variability in anti-*H. pylori* antibody patterns reported in the literature (Karczewsa *et al.*, 1996; Karvar *et al.*, 1997; Nilsson *et al.*, 1997; Blanchard *et al.*, 1999), which probably reflects differences in host and bacterial

factors. Antibodies against various *H. pylori* proteins can be detected in patient sera (Aucher *et al.*, 1998) but the identity of many of these proteins remains to be established. Immunodominant proteins of *H. pylori* that have been well-characterized include the urease subunits (Ferrero *et al.*, 1994), the vacuolating cytotoxin (VacA) and cytotoxin-associated protein (CagA) (Covacci *et al.*, 1991), proteins involved in flagella structure (Luke and Penn, 1995) and other surface-associated antigens (Guruge *et al.*, 1990).

Cytotoxic strains expressing the CagA antigen and the vacuolating cytotoxin (VacA) are associated with a more severe disease (van Doorn *et al.*, 1998) and both of these antigens elicit a specific antibody response during infection. Studies of infected patient responses to *H. pylori* antigens have shown that several immunodominant antigens, including CagA, are not recognised by non-infected patients (Crabtree *et al.*, 1994) and are therefore useful for diagnosis. Many of the diagnostic tests for *H. pylori* infection involve enzyme-linked immunoabsorbant assays (ELISA) using a variety of antigenic preparations. The specificity of these tests is often hampered by cross-reactivity (van Zwet and Megraud, 1998). Thus, the identification of specific major antigens is fundamental for the development of improved diagnostic tests. Immunoblotting techniques also used for the diagnosis of *H. pylori* infection in humans are recommended when the outcome of the ELISA is equivocal (Nilsson *et al.*, 1997).

Like other gram-negative bacteria, *H. pylori* shed part of their outer membrane as vesicles under certain growth conditions (reviewed in Beveridge, 1999). Immunisation with *H. felis* outer membrane vesicles (OMVs) and cholera toxin (CT) as an adjuvant, can protect mice against subsequent challenge with *H. felis* (Keenan *et al.*, 1997). Moreover, protection correlates with an antibody response to an 18 kDa *H. felis* outer membrane component (Keenan *et al.*, 1995). Immunisation of mice with *H. pylori* OMVs and CT also elicits a serum IgG response to a similar size outer membrane antigen from *H. pylori* (Keenan *et al.*, 1998). A monoclonal antibody raised against the this OMV antigen from *H. pylori* (Keenan *et al.*, 1998) was made available as part of the present study.

This study describes a preliminary investigation into the serological response to antigens derived from a *H. pylori* New Zealand clinical isolate (NZCH1) (Mooney *et*

al., 1991). This *H. pylori* strain was shown to be *cag*A+ *vac*A+ by Southern blotting, and the phenotypic expression of these gene products confirmed by Western blot analysis of protein prepared from cell lysates of *H. pylori*. A genomic library of *H. pylori* NZCH1 was constructed in the expression vector Lambda Zap (Stratagene) and the expression library was screened with the monoclonal antibody as part of a collaborative project. This identified the antigen as Lpp20, a previously characterised lipoprotein from *H. pylori* (Kostryznska *et al.*, 1994), recently shown to be a protective antigen in a murine model of *H. pylori* infection (Hocking *et al.*, 1999).

3.3 Materials and Methods

3.3.1 Construction of a *H. pylori* expression library

3.3.1.1 Digestions and Ligations

A clinical *H. pylori* isolate, designated NZCH1, originally isolated from a patient undergoing upper gastrointestinal endoscopy (Mooney *et al.*, 1991), was chosen as the source of genomic DNA for construction of the *H. pylori* expression library. Five microlitres of the Lambda ZAP vector (Statagene) was digested with 10 units of *Bam* HI and dephosphorylated with calf intestinal alkaline phosphatase (CIAP). After the addition of 5 mM EDTA, 0.5% SDS and 50 μg/ml Proteinase K, the vector was incubated at 56°C for 30 mins. Prior to the ligation, the vector was extracted three times with phenol:chloroform and ethanol precipitated (Chapter 2.4.1). Approximately 5 μg of genomic DNA isolated from *H. pylori* NZCH1, (Chapter 2.3.3) was partially digested with *Sau* 3A (0.3 units) for 10 mins at 37°C. Following electrophoresis, DNA fragments between 2 and 10 kilobase pairs (kbp) were excised from a 1% low melting point agarose and purified using GELase (Epicentre Technologies) according to the manufacturer's instructions. The *H. pylori* DNA fragments were ligated into the *Bam* HI site of Lambda Zap at 4°C for 48 hrs.

3.3.1.2 Packaging of the ligation

The recombinant Lambda were packaged using a MaxPlax Packaging Extract (Epicentre Technologies, Wisconsin) according to the manufacturer's instructions. Five microlitres of the ligation mix was added to 50 μl of packaging extract and incubated at

RT for 2 hours. Following the addition of 500 µl of phage buffer (10 mM Tris-HCl (pH 8.3), 100 mM NaCl, 10 mM $MgCl_2$) and 20 µl of chloroform, the packaged phage (1 µl) were used to transfect *E. coli* XL1-blue (OD_{600} 0.5) by incubating the cells with the phage at 37°C for 30 mins. The phage were plated by pouring the cells onto an LB plate after the addition of 3 ml of melted top agar containing 10 mM $MgSO_4$, 2.5 mM IPTG and 4 mg/ml X-Gal for determination of the number of recombinant Lambda particles. Plates were incubated overnight at 37°C. In addition, serial dilutions of the packaged phage were made and used to transfect *E. coli* XLI-blue as above to determine the titer of plaque-forming units (PFU) per millilitre.

3.3.1.3 Amplification of library

A culture of *E. coli* XLI-blue was grown to an OD_{600} of 1.0 in LB broth supplemented with 0.2% maltose and 10 mM $MgSO_4$. Aliquots of the packaged phage were mixed with 600 µl of host cells at an OD_{600} of 0.5 and incubated at 37°C for 15 mins. Five ml of melted top agar was added to each aliquot of infected bacteria and poured over LB agar, and the plates incubated at 37°C for 6 – 8 hours. The plates were then overlayed with 8 ml of SM buffer (0.1 M NaCl, 10 mM $MgSO_4.H_2O$, 50 mM Tris-Cl (pH 7.5), 2% gelatin) and stored at 4°C overnight to allow the phage to diffuse into the buffer. The bacteriophage suspension was recovered from the plates, pooled and chloroform added to a 5% final concentration. After thorough mixing, the suspension was incubated at RT for 15 mins, and the cell debris removed by centrifugation. Chloroform was added to the supernatant to a final 0.3% and 7% of dimethylsufoxide was added before storing at –70°C.

3.3.2 Immunoscreening

3.3.2.1 Immunoscreening of *H. pylori* protein with patient sera

Lysate protein was prepared from *H. pylori* Royal Perth Hospital strain 13487 (NZRM Accession No. 2925, Type Strain NCTC 11637) and *H. pylori* NZCH1 and quantitated as previously described (Chapter 2.9). For immunoscreening, 50 µg of *H. pylori* 11637 protein was electrophoresed in quadruplicate alongside a protein marker on a 10% SDS-polyacrylamide gel and blotted onto PVDF membrane as described in Chapter 2.10.

Following incubation in blocking solution at RT for 2 hrs, the membrane was divided into two and incubated with sera from either an infected (PS025) or uninfected (PS001) patient. Sera was collected as described (Chapter 2.11) and used at a dilution of 1:200. For patient details refer to Table 2.3. Membranes were incubated in sera overnight at 4°C prior to washing with PBS-T. Following the washes, the blots were incubated with anti-human IgG conjugated to horseradish peroxidase (POD) (Boehringer Mannheim) diluted 1:5,000 for 30 mins at RT and developed by autoradiography by exposure to X-ray film for 1 min as described in Chapter 2.11. The positions of the immunoreactive bands revealed by the patient's sera were assessed with a calibrating curve constructed by plotting the distances of migration of the protein standards visible on the membrane.

3.3.2.2 Immunoscreening of the *H. pylori* expression library for an 18 kDa antigen

Two microlitres of packaged phage from the amplified Lambda library was used to transfect *E. coli* XL1-blue (OD_{600} 0.5) by incubating the cells with the phage at 37°C for 30 mins. The phage were plated by pouring the cells onto a LB plate after the addition of 3 ml of melted top agar containing 0.2% maltose and 10 mM $MgSO_4$. Assuming a genome size of 1,667,867 bp and an average insert of 5,000 bp, then the number of independent recombinant plaques that must be recovered in the Lambda vector in order to have a 99% probablility of detecting a single gene is 1,471 (Sambrook *et al.*, 1989). Discs of Hybond-C extra hybridisation transfer membrane (Amersham) were soaked in 10 mM IPTG and placed onto the plates following overnight incubation at 37°C. The plates were left to incubate at 37°C for a further 6 hours to allow for induction of any β-galactosidase fusion proteins, and then placed at 4°C overnight. The membranes were removed and placed into 2% blocking solution at room temperature for Western blot development. The membranes were incubated in blocking solution for 2 hrs at RT followed by incubation in a monoclonal antibody raised to an *H. pylori* 18 kDa antigen diluted 1:100 for 2 hours at RT. Following washing with PBS-T, membranes were incubated with the goat anti-mouse IgG alkaline phosphatase conjugate (Sigma, St. Louis) at a dilution of 1:1,000 at RT for 1 hr. The membranes were washed with PBS-T and positive plaques detected by the antibody were visualised by the addition of the substrates BCIP and NBT as described (Chapter 2.10.3).

3.3.3 Polymerase chain reaction

The polymerase chain reaction was used to amplify the urease B subunit (*ure* B), flagellin subunit A (*fla* A), vacuolating gene (*vac* A) and cytotoxin-associated gene (*cag* A). *H. pylori* genomic DNA was isolated from *H. pylori* type strain 17874 (Chapter 2.2.3) and 100 ng used as a template in the PCR. The oligonucleotide primers are listed in the following table:

Table 3.1: Oligonucleotide primers designed to amplify *H. pylori* gene sequences

***H. pylori* gene**	**Olilgonucleotide Sequence (5'-3')** (forward primer) (reverse primer)	**Product Size**	**Reference**
ure B	AAAAGATCTAAGGAGTAAGAAATGAAAAAGATT AAAGAATTCTAGAAAATGCTAAAGAGTTGC	1742 bp	This study
fla A	ATGGCTTTTCAGGTCAATAC GCTTAAGATATTTTGTTGAAC '	1499 bp	Bukanov *et al.* (1994)
vac A	GCTTCTCTTACCACCAATGC TGTCAGGGTTGTTCACCATG	1160 bp	Xiang *et al.* (1995)
*cag*A	AGTAAGGAGAAACAATGA AATAAGCCTTAGAGTCTTTTTGGAAATC	1350 bp	Xiang *et al.* (1995)

The PCR products were amplified using Taq DNA polymerase (Boehringer Mannheim) under standard conditions (Chapter 2.6) The PCR reaction consisted of an initial denaturation step of 5 mins at 95°C, followed by 25 cycles of denaturation at 95°C for 30 secs, annealing at 55°C for 30 secs and an extension step at 72°C for 40 secs. Products were visualised on a 1.0% TBE agarose gel as previously described (Chapter 2.4.2).

3.3.4 Southern blotting

3.3.4.1 Southern blot of *H. pylori* genomic DNA

Genomic DNA from *H. pylori* NZCH1 and *H. pylori* 17874 was isolated (Chapter 2.4.1) and 5 μg digested with *Hin*d III at 37°C for one hour. Digested fragments were electrophoresed on a 1% agarose gel and transferred to Hybond nylon membrane

(Amersham) by capillary action as previously described (Chapter 2.8.2). The *cag* A and *vac* A genes were amplified by PCR as described above and purified from a 1% TAE agarose gel using a Bresaclean purification kit (Bresatec, Aus). The purified PCR product from the *cag* A gene was then DIG-labelled and quantitated (see Chapter 2.8.1) and 25 ng probe per ml hyridisation solution used in the Southern blot experiments. Following prehybridisation, membranes were hybridised overnight at 42°C and developed with anti-DIG antibody conjugated to alkaline phosphatse (Chapter 2.8.2). The *vac* A gene was radiolabelled with ^{32}P[dCTP] and 50 µl of labelled probe used in the Southern blot and the results visualised by autoradiography as previously described (Chapter 2.8.2.1).

3.3.4.2 Plaque hybridisation of the *H. pylori* expression library

Five microlitres of phage stock was diluted in 995 µl of phage buffer and 100 µl aliquots added to *E. coli* XLI-blue (OD_{600} 0.6) and incubated for 20 mins at 37°C before plating onto LB media as described above (3.3.3.2). The resultant plaques were transferred to nylon disc membranes in duplicate as follows. The plates were incubated overnight at 37°C and chilled for 2 hours at 4°C. The plaques were then transferred onto Hybond-N+ Nucleic Acid Transfer Membrane discs for 2 minutes. The membranes were then denatured by submerging in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 2 minutes and then neutralised by submerging the membrane in neutralisation solution (1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0)). The DNA was fixed by exposure to UV light for 2 minutes. The 1742 bp *ure*B and 1499 bp *fla*A PCR product was generated using the appropriate primers as described above (3.3.4), radiolabelled with ^{32}P[dCTP] and used in the hybridisation reaction at 65°C as described elsewhere in Chapter 2.8.2. Positive plaques detected by the probe were visualised by autoradiography.

3.3.5 Analysis of Lambda phage

3.3.5.1 Storage of plaques

Positive plaques, detected by autoradiography or colorimetric development, were selected by aligning the membrane with the original plate. Corresponding plaques were taken as plugs of agar and transferred to 500 µl of SM buffer containing 20 µl of

chloroform. The suspension was then vortexed to release the phage particles and stored at 4°C.

3.3.5.2 Conversion of positive plaques to phagemids

The *in vivo* excision and recirculisation of the cloned insert to form a phagemid containing the *H. pylori* insert was carried out by co-infecting the phage stock with *E. coli* XLI-blue and ExAssist helper phage (>1x10^6pfu/ml) according to the manufacturer's instructions. The phage stock (250 µl) was added to 200 µl of XLI-blue cells (OD_{600} 1.0) along with 1 µl of ExAssist helper phage and incubated at 37°C for 15 mins. After the addition of 3 ml LB broth, the mixture was incubated a further 2 hrs at 37°C and then heated to 70°C for 15 mins. Following centrifugation at 4,000 g for 15 mins, the supernatant was collected and stored at 4°C. To plate the excised phagemids, 10 µl of phage supernatant was added to 200 µl of *E. coli* XLOR cells (OD_{600} 1.0) and incubated at 37°C for 15 mins. Following the addition of 300 µl of LB broth, the cells were incubated a further 45 mins at 37°C and 200 µl plated onto LB plates containing 50 µg/ml of kanamycin.

3.3.5.3 Sequence analysis of phagemid inserts

The cloned insert contained within the lambda vector from the positive plaques was excised *in vivo* and recircularised to form a phagemid containing the cloned insert as described above. Phagemid DNA was prepared from *E. coli* XLOR using the Bresatec Plasmid Isolation Kit (Bresatec, Australia). The *H. pylori* DNA inserts in the phagemids were analysed by sequencing across the cloning junctions using the universal primers T3 and T7. Sequencing was carried out by Massey University DNA Analysis Service. The DNA sequences generated were compared to the published TIGR sequence of *H. pylori* strain 26695 and the genes encoded in each phagemid identified.

3.3.6 Cloning and expression of recombinant *H. pylori* genes

3.3.6.1 Cloning of HP ORFs into expression vectors

Oligonucleotide primers were designed to amplify ORFs HP1456 and HP1457 based on the sequence from the published genome sequence (Tomb *et al.*, 1997). The primers

were designed to amplify the ORF devoid of its signal sequence, with a *Bam* HI site incorporated into the 5' end and a *Eco* RI site at the 3' end. Primers were designed as described in Chapter 2.6, and tested for their suitability in the PCR reaction using Amplify version 1.2. The primer sequences, with the incorporated restriction site shaded, are listed in Table 3.2. Genomic DNA prepared from *H. pylori* 17874 was used as the template in the PCR. The PCR products were amplified using Pwo Taq polymerase (Boehringer Mannheim), a high fidelity Taq polymerase with 3'-5' exonuclease (proofreading) activity that amplifies DNA with tenfold greater accuracy than common Taq polymerase. PCR products were amplified under standard conditions

Table 3.2: Oligonucleotide primers designed to amplify HP1456 and HP1457

Primer Name	Oligonucleotide sequence (5'–3') (forward primer) (reverse primer)	Reference
HP 1456/B	CTTTAGGATCCGTGGGTTGCTGAAG	This study
HP 1456/E	TATTTGAATTCAAAACATACGCTTA	This study
HP 1457/B	TCGTAGGATCCAGCCATGCC	This study
HP 1457/E	AAGGCGAATTCTTAAAACCCT	This study

(Chapter 2.6). The PCR reaction was as described in section 3.3.4, with an adjusted annealing temperature of 60°C. Products were visualised on a 1% TAE agarose gel and purified using QIAGEN gel purification kit (QIAGEN, Aus). Purified products were then digested with the restriction enzymes *Bam* HI and *Eco* RI and cloned into the *Bam* HI- *Eco* RI sites of the expression vectors, pPROex HTb (Life Technologies) and pGEX-6P-3 (Pharmacia Biotech) (Figure 3.1). Recombinant plasmids were purified and transformed into *E. coli* for expression studies. All cloning and transformation techniques were carried out using standard procedures (Chapter 2.4 & 2.5).

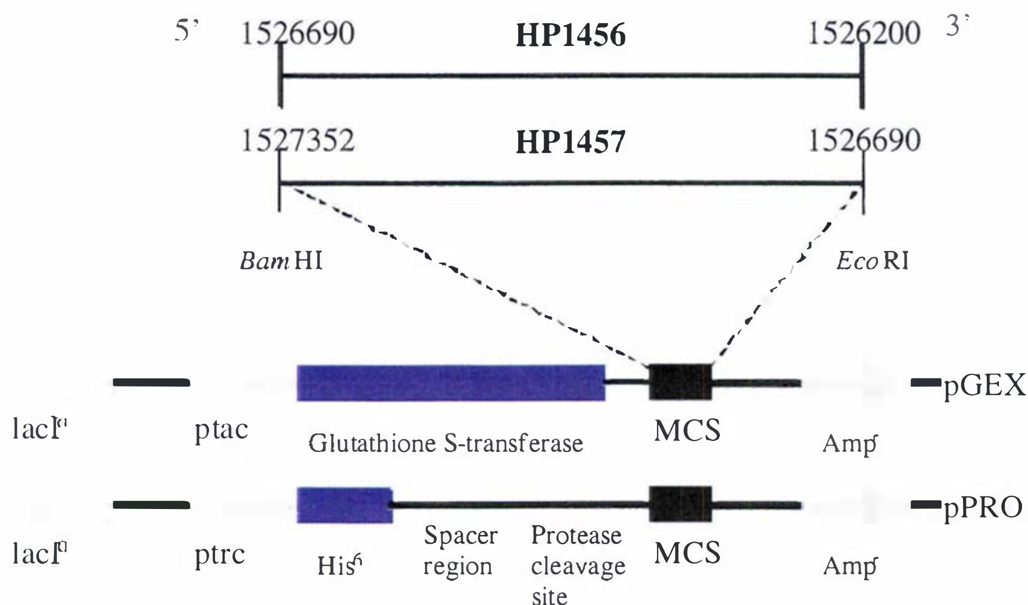


Figure 3.1: Schematic representation of cloning of HP1456 and HP1457 into two expression vectors, pGEX-6P-3 and pPROEX HTb showing the major features of each vector. Numbers at the 5' and 3' end refer to the position of the amplified fragments on the published genome (Tomb *et al.*, 1997).

3.3.6.2 Expression of the recombinant proteins in *E. coli*

Maximum expression of the recombinant proteins was determined by induction trials with varying amounts of isopropyl-β-δ-thiogalactopyranoside (IPTG) over a time course of 1 hour and overnight induction. IPTG was added into the culture medium once the *E. coli* cells had reached mid-log growth phase (OD_{600}=0.5-1.0). Following induction, the cells were harvested by centrifugation at 14,000 rpm and resuspended in 20 mM Tris pH 8, 10 mM $MgCl_2$. Total protein was electrophoresed on SDS-PAGE gels and stained with Coomassie as previously described (Chapter 2.10). For Western blotting, the separated protein was transferred to PVDF membrane and developed as described in section 3.3.3.2, using the monoclonal antibody to the 18 kDa antigen as the primary antibody, with a one hour incubation at RT.

3.4 Results

3.4.1 Differential immunoscreening of *H. pylori* total protein

The *Helicobacter pylori* type strain NCTC 11637 was grown under conditions described previously (Chapter 2.2). Small transparent glassy colonies were observed after 72 hours of incubation. The bacterial cells comprising the colonies were confirmed to be Gram negative and subsequently tested for urease activity. Following innoculation of test agar with the bacteria, a pH change, indicated by the development of a pink color, was observed after 10 mins. Total *H. pylori* protein was prepared from pooled colonies and separated by SDS-PAGE. The protein profiles of the *H. pylori* type strain (NCTC 11637) and clinical isolate NZCH1 were compared by Coomassie staining. Both isolates showed very similar protein profiles, with major bands conserved. The strongest bands corresponded to apparent molecular weights (mw) of approximately 15, 20, 25, 55, 60, 66 and 90 kDa, with a number of minor protein bands observed, particularly in the range between 45 and 87 kDa (Figure 3.2). The sera from

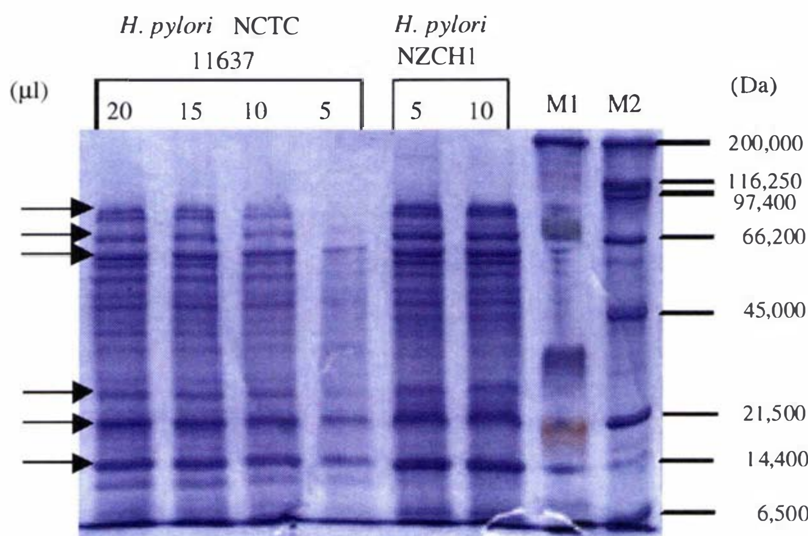


Figure 3.2: Comparison of protein profiles from a *H. pylori* type strain NCTC 11637 and the human clinical isolate (NZCH1) used in this study. Lysate protein (5, 10, 15 or 20 µl volumes) was prepared as previously described (Chapter 2.10) and separated by electrophoresis on a 10% SDS-PAGE gel. Following electrophoresis, the proteins were visualised by Coomassie blue stain alongside two protein standard markers. M1: Kaleidoscope marker (BIORAD); M2: BIORAD broad range marker. Arrows refer to positions of major protein bands common between the two strains.

an *H. pylori*-infected and non-infected patient was used in immunoblot experiments to probe total protein. Eight reactive bands in the molecular weight range of 25 – 150 kDa were observed in all subjects irrespective of *H. pylori* status. A number of immunoreactive bands in the molecular weight range of 14 – 128 kDa were found only in patients with positive *H. pylori* status (Figure 3.3). The immundominant proteins detected only by the infected patient sera were of apparent molecular weight (mw) 130, 90, 75, 55, 50, 42, 36, 35, 25 and 14 kDa. The protein bands at 130 kDa and 90 kDa are consistent with the sizes of the CagA and VacA proteins. The results of this screen indicated that there are a number of *H. pylori* antigens that only antibodies from infected patients recognise, and therefore may be involved in the pathogenesis of infection.

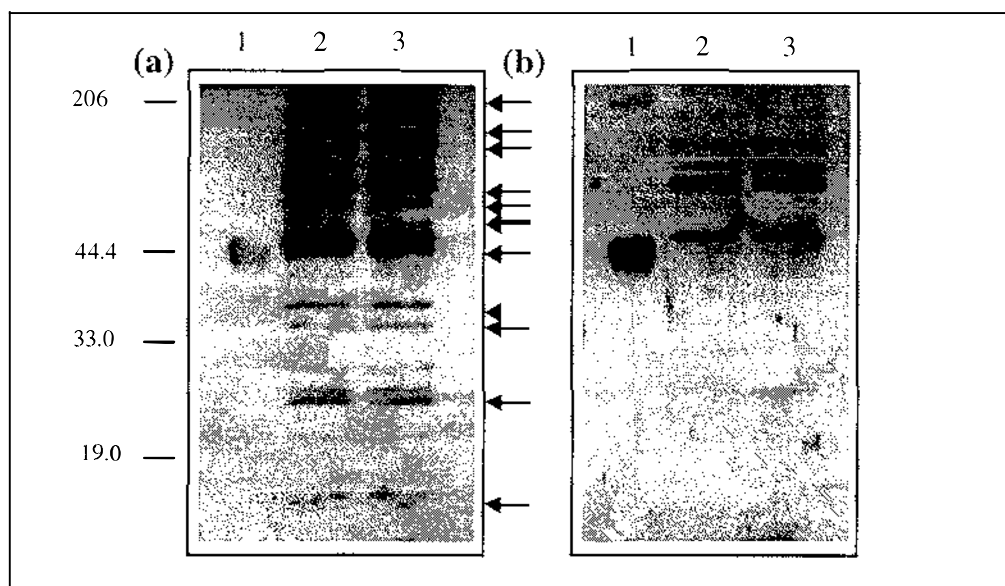


Figure 3.3: Autoradiograph of Western blot analysis of *H. pylori* NZCH1 lysate protein screened with sera from an *H. pylori* sero-positive (a) and sero-negative (b) patient. Lane 1, Kaleidoscope standard marker; lane 2 and 3, *H. pylori* lysate protein. Numbers on the left refer to molecular weight (mw) size markers (kDa). Arrows refer to the unique protein bands detected by the sero-positive patient sera.

3.4.2 Analysis of *H. pylori* NZCH1 genotype and phenotype for use in a *H. pylori* gene expression library

Type I strains of *H. pylori* contain the genes *vac*A and *cag*A, and produce a vacuolating cytotoxin (VacA). The majority of ulcerogenic strains of *H. pylori* have been found to contain these genes, although combinations of this genotype/phenotype exist (Xiang *et*

al., 1995). To confirm *H. pylori* NZCH1 was a Type I strain, the genes encoding the VacA and CagA proteins were amplified by PCR from genomic DNA isolated from the *H. pylori* clinical isolate NZCH1 and the type strain NCTC 11637 using published primers as described above (3.3.4). The PCR reaction yielded the expected products of 1350 bp (*cag*A) and 1160 bp (*vac*A) (Figure 3.4 (1a) and (2a)). The PCR products were either DIG-labelled or ^{32}P[dCTP]-labelled and used as probes in a Southern blot against *Hin*d III digested genomic DNA. The results of these blots confirmed the presence of these two genes in the genomic DNA of *H. pylori* isolate NZCH1 (see Figure 3.4).

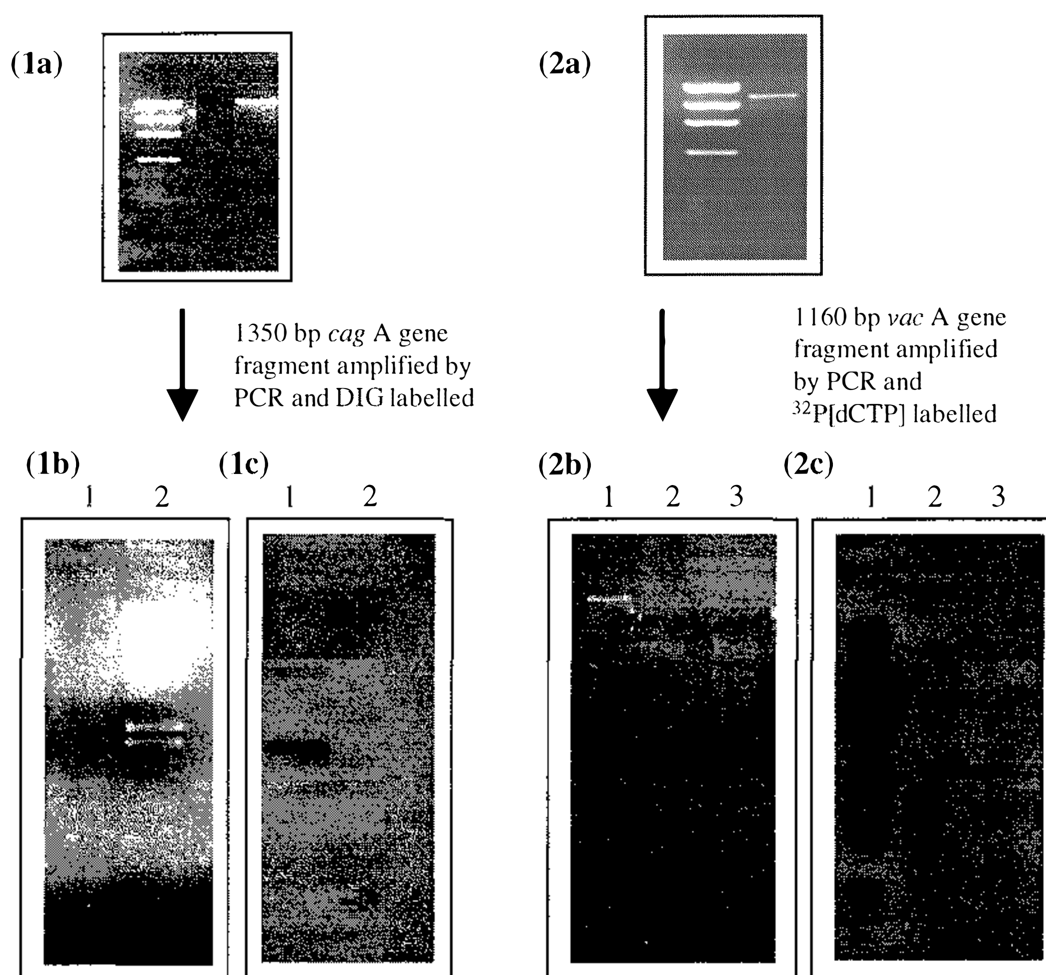


Figure 3.4: Southern blot experiments to detect the *cag* A (1) and *vac* A (2) genes in *H. pylori* genomic DNA. 1a and 2a: Agarose gel of PCR product amplified from the *cag* A and *vac* A genes from *H. pylori* 17874 genomic DNA; 1b and 2b: Agarose gel of *H. pylori* genomic DNA digested with the restriction enzyme *Hin*d III and separated on a 1% agarose gel; 1c and 2c: Autoradiograph of Southern blot. (1) genomic DNA was probed with DIG-labelled *cag* A gene product; lane 1, *H. pylori* NZCH1; lane 2, DNA marker. (2) genomic DNA was probed with 32P[dCTP]-labelled *vac* A gene product; lane 1, DNA marker; lane 2, *H. pylori* 17874, lane 3, *H. pylori* NZCH1.

In addition, a Western blot was performed on total protein from *H. pylori* isolate NZCH1 using a polyclonal antibody raised against the VacA and CagA proteins. Lysate protein was prepared from both the clinical isolate (NZCH1) and *H. pylori* type strain 17874. An *E. coli* lysate sample was used as a negative control. The blot was probed with anti-VacA (1:5000) or anti-CagA (1:5000) antibodies (kindly supplied by P.W.O'Toole, Massey University) and detected using colorimetric development (see Chapter 2.10.3). The presence of the CagA protein was detected as a band $>$ 120 – 128 kDa and the VacA at approximately 87 kDa, (Figure 3.5) providing further confirmation of a Type I phenotype. This result demonstrated that the strain expresses both the cytotoxin and cytotoxin-associated protein when cultured *in vitro*.

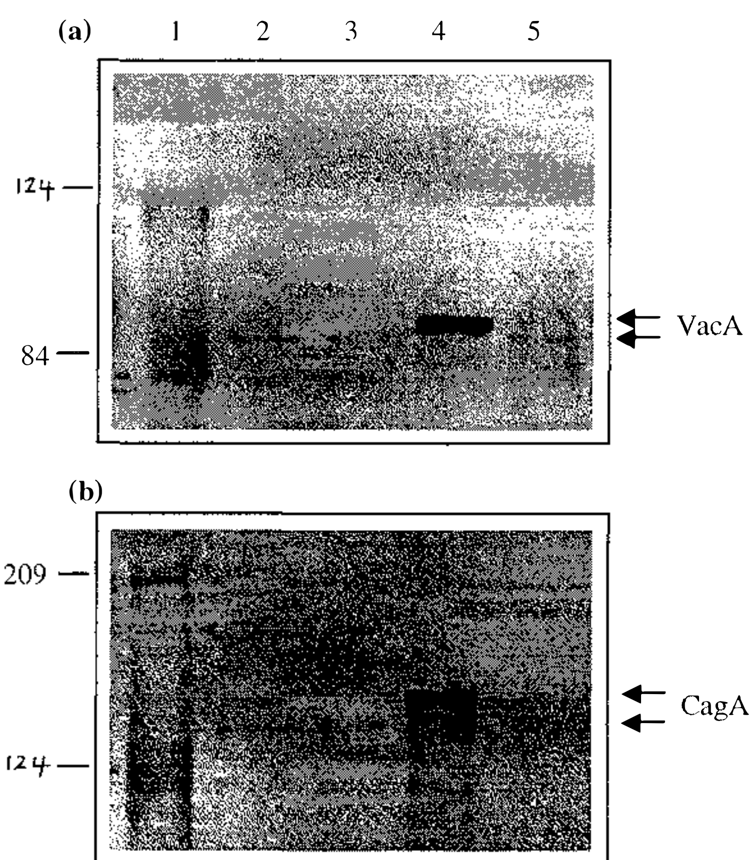


Figure 3.5: Western blots of *H. pylori* and *E. coli* lysate protein screened with (a) anti-VacA antibody and (b) anti-CagA antibody. Lane 1, Kaleidoscope marker; lanes 2 and 3, *H. pylori* NZCH1 lysate protein; lane 4, *H. pylori* 11636 lysate protein; lane 5, *E. coli* DH10B lysate protein. Positive protein bands were detected by colorimetric development.

3.4.3 Construction of a genomic *H. pylori* expression library

An *H. pylori* expression library was constructed using the Lambda Zap Express vector (Statagene). A color assay was used to determine the ratio of recombinants to non-recombinants by plating the library on IPTG and X-Gal. Non-insert background plaques, in which there is a functional *lac*Z gene generate a blue colour when plated on these substrates. In total, there were approximately 50 blue plaques for every 1000 clear plaques, indicating that 95% of the library was recombinant. Titering of the packaged phage generated the following plaque numbers:

Dilution	10^{-1}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
No. plaques	confluent	900-1000	80	9	1

Using these figures, the titer was calculated to be 1.1 x 10^8 pfu/μg

3.4.4 Southern blot screening of the *H. pylori* expression library for *H. pylori* genes

To gain an indication that the expression library was representative of the *H. pylori* genome, the library was screened for two known *H, pylori* genes. Genomic DNA from *H. pylori* 17874 was used as a template to PCR amplify the *H. pylori* genes encoding the urease B subunit and flagellin A subunit (section 3.3.5). The PCR generated the expected products of 1742 bp for the *ure*B gene and 1499 bp for the *fla*A gene (Figure 3.6(a)) and sequencing of the PCR products confirmed these sequences. The resultant PCR products were purified and radiolabelled for use as a probe in screening the Lambda library. Assuming a genome size of 1,667,876 bp and an average insert of 5,000 bp, then the number of independent recombinant plaques that must be recovered in the Lambda vector to have a 99% probability of detecting a single gene is 1,471 (Sambrook *et al.*, 1989). The recombinant phage were plated at a density of 1,000 pfu per plate for screening, and following three hours exposure, 40–50 strong positive signals were detected on each membrane. An example of this screening is shown in Figure 3.6 (b). Three positive plaques for the urease gene were selected and converted to phagemids. Phagemid DNA was prepared and digested with the restriction enzymes

Pst I and *Hin*d III to excise the insert, and separated on an agarose gel followed by transfer to membrane for Southern blot screening. The original radiolabelled PCR product was used as a probe against the Southern blot and the presence of this gene was confirmed by a positive signal with the corresponding phagemids (data not shown). This screening indicated that the *H. pylori* genome was representative and suitable for screening for immunoreactive antigens.

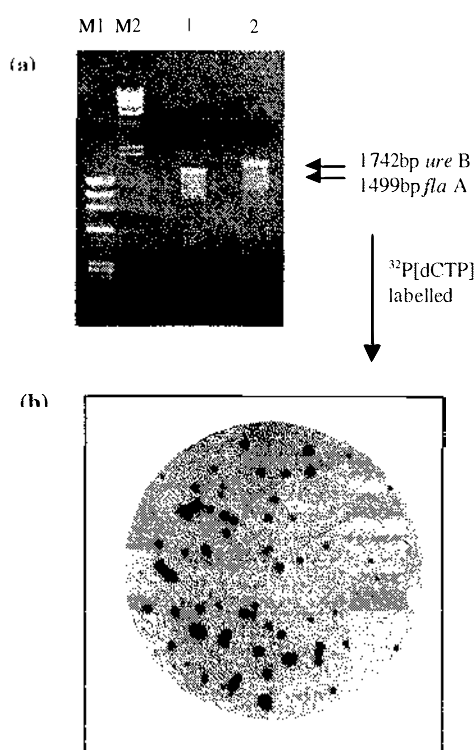


Figure 3.6: Southern blot screening of the *H. pylori* expression library. (a) Agarose gel of PCR products amplified from the *fla*A and *ure*B genes from *H. pylori* 17874. (b) Autoradiograph of Southern blot following screening of the expression library with radiolabelled *ure*B sequence after three hours exposure.

3.4.5 Plaque immunoblotting of the *H. pylori* expression library using a monoclonal antibody raised against the immunogenic 18 kDa protein

The 18 kDa antigen is a component of an outer membrane vesicle preparation from *H. pylori*. A monoclonal antibody specific for this antigen was made available for this study (Keenan *et al.*, 1998). Immunoscreening of the expression library was performed as described in the materials and methods (3.3.3.2). The *H. pylori* expression library was transferred to Hybond-C extra membrane discs and screened with the monoclonal antibody raised to the 18 kDa *H. pylori* outer membrane protein. Eight strongly positive plaques were selected and their reactivity confirmed with secondary screening. An example of this is presented in Figure 3.7. The *H. pylori* DNA from each of the eight

phage was then excised and recirculised to form a phagemid containing the *H. pylori* insert DNA as described in the materials and methods (3.3.6.2).

Figure 3.7: Secondary screening of the *H. pylori* Lambda library with a monoclonal antibody raised to an 18 kDa antigen from membrane vesicles of *H. pylori.* Positive plaques were detected on the membrane by colorimetric development.

3.4.5.1 Sequence analysis of phagemid inserts

Restriction digests of the selected positive phagemids revealed that six contained an identical insert of approximately 2 kb while the remaining two contained inserts of approximately 2.5 kb (data not shown). Sequence analysis of these phagemid inserts revealed that they all mapped to the same region of the *H. pylori* genome. The locus is diagrammatically represented in Figure 3.8 and encompasses the promoter and start

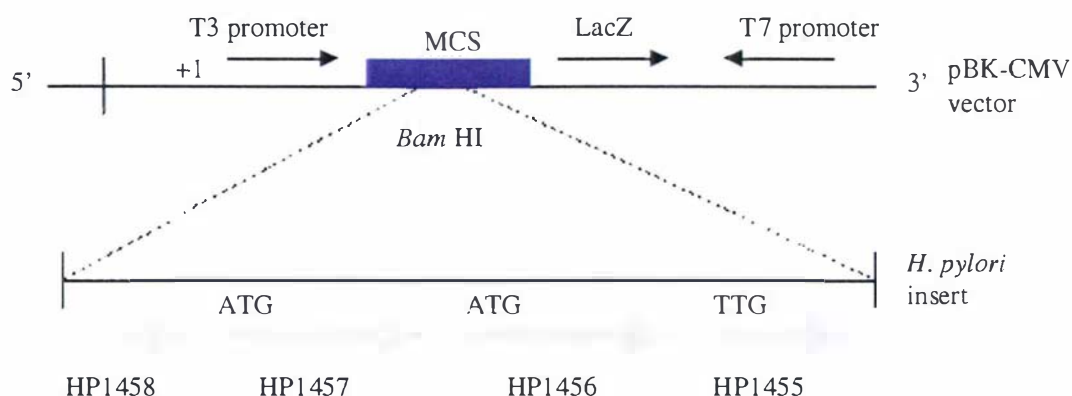


Figure 3.8: Schematic representation of the *H. pylori* insert sequence from a positive phagemid following screening with an 18 kDa monoclonal antibody. Met: start methionine of β-galactosidase (β-gal); MCS: multiple cloning site. HP numbers refer to the number of the ORF designated in the published genome (Tomb *et al.*, 1997).

codons of three genes (HP1455, HP1456 and HP1457). Only two of these three genes (HP1456 and HP1457) contained a putative signal sequence, and was therefore a

candidate to encode an outer vesicle membrane protein. HP1456 and HP1457, when translated from the putative cleavage point produce proteins of approximately 19.11 kDa and 23.31 kDa . Although the predicted size of the gene product indicated that the HP1456 ORF was likely to encode the 18 kDa antigen detected by the antibody, further experiments were required to confirm this. Since HP1455 did not appear to contain a signal sequence, and encoded a smaller protein of 14 kDa, this gene was not included in the following experiments.

3.4.6 Identification of an immunogenic 18 kDa antigen

To investigate which ORF (HP 1456 or HP 1457) was encoding the 18 kDa antigen, the ORFs of each were cloned and the protein expressed as a recombinant fusion protein. Oligonucleotide primers were designed to amplify a truncated ORF devoid of the signal sequence. PCR was carried out as described (Section 3.3.6) and the expected PCR products were purified from an agarose gel, digested with the appropriate restriction enzymes and cloned into the compatible sites of the expression vectors pPROEX HTb (Life Technologies, USA) and pGEX-6P-3 (Pharmacia Biotech, USA) (see Figure 3.1). Expression of the recombinant fusion protein was induced by the addition of 0.5 and 1mM IPTG and analysed by SDS-PAGE at various time intervals. The induction trial for HP 1457 – His^6 is shown in Figure 3.9 and was similar for HP 1456 – His^6.

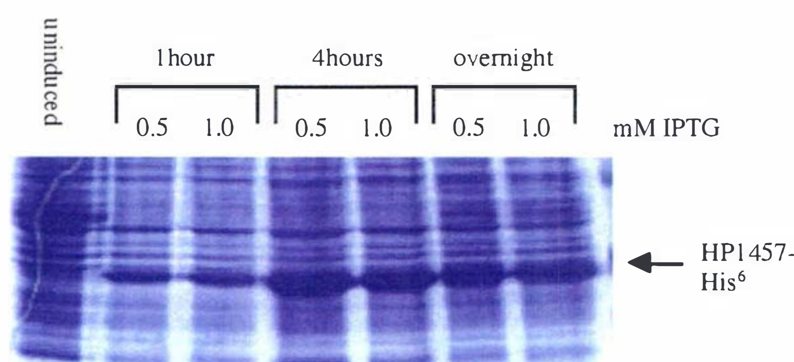


Figure 3.9: Expression of HP1457 in pPROEX HTb resulting in a 27.8 kDa fusion protein consisting of HP 1457 and a six Histidine tag (6.8 kDa) for affinity purification. Expression of the recombinant protein was induced with both 0.5 mM and 1 mM IPTG and the cells harvested at time intervals following 1 hour, 4 hours and overnight induction. Twenty microlitres of resuspended cells from the uninduced, 1 hour and 4 hour induction were electrophoresed on a 12.5% SDS-PAGE gel along with 10 µl of overnight sample and stained with Coomassie.

Expression of HP 1456 and HP 1457 in pPROex HTb resulted in the production of a fusion protein of 23.6 kDa and 27.8 kDa respectively, with the N-terminal signal sequence replaced with a string of six Histidines, a spacer region and rTEV protease cleavage site (Figure 3.10 (a)). Similarly, induction of expression in pGEX-6P-3 resulted in a fusion protein of 42.8 kDa for HP1456 and 47.0 kDa for HP1457 (Figure 3.10 (a)) with the N-terminal being replaced by Glutathione S-transferase and a recognition site for the protease PreScission™. Total protein was prepared from cultures of cells containing each construct and separated on 12.5% SDS-PAGE gel and transferred to PVDF for Western with the monoclonal antibody to the 18 kDa antigen.

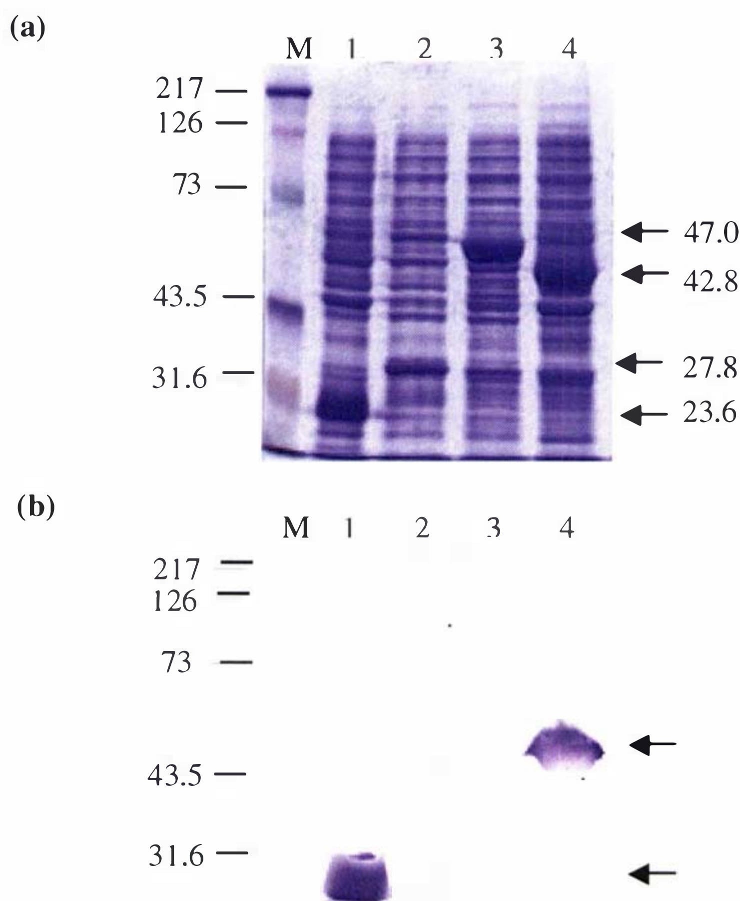


Figure 3.10: Identification of HP1456 with an anti-18 kDa monoclonal antibody. (a) Coomassie stained gel. *H. pylori* proteins HP1456 and HP1457 were expressed as fusion proteins as both Histidine tagged proteins and GST tagged protein, and electrophoresed on a 15% SDS-PAGE gel. (b) Western blot. Proteins were transferred to PVDF membrane and screened with a monoclonal antibody. M, Kaleidoscope marker (BIORAD); lane 1, HP1456-His; lane 2, HP1457-His; lane 3, HP1457-GST; lane 4, HP1456-GST.

A positive reaction was seen with both HP1456 fusion proteins, but no reaction was observed with either HP1457 proteins, confirming that the HP1456 ORF encodes the 18 kDa antigen (Figure 3.11 (b)).

3.5 Discussion

H. pylori possesses a number of antigens that induce an immune response in infected patients (Andersen *et al.*, 1995). The initial characterisation of *H. pylori* protein profiles and the serological response to these was performed primarily to assess the feasibility of using sera as a screening tool to identify *H. pylori* antigens. Early reports on whole-cell profiles of *H. pylori* showed that the bacterium constitutes a relatively homogeneous species (Perez-Perez and Blaser, 1987). SDS-PAGE analysis of protein profiles from different *H. pylori* strains have demonstrates a number of common major bands. Seven major bands of molecular weights (mw) 62,000, 56,000, 53,000, 48,000, 44,000, 29,000 and 26,000 are consistently present among strains, with considerable variation among the minor bands (Perez-Perez and Blaser, 1987; Dunn, 1993). There also appears to be a high degree of conservation among outer membrane proteins (OMPs) of *H. pylori*, with major bands being found at 61, 54, 46, 40, 30 and 19 kDa (Drouet *et al.*, 1991). More recent studies have divided *H. pylori* antigens into three major groups based on their molecular weight (Karvar *et al.*, 1997). The low molecular mass proteins (LMMPs) include the 30 kDa urease antigen (UreA), while the high-molecular mass proteins (HMMPs) include the 87 kDa vacuolating cytotoxin (VacA) and the 128 kDa cytotoxin-associated protein (CagA) (Karvar *et al.*, 1997)

The two *H. pylori* strains examined in this study showed consistent results with the earlier reports on *H. pylori* antigenic profiles, with some variations on the estimated molecular weights. The major bands seen at 20 and 25 kDa may correspond to the 19 kDa outer membrane protein (Drouet *et al.*, 1991) and the species specific 26 kDa antigen (O'Toole *et al.*, 1991) previously described. Hawtin *et al.* (1990) demonstrated that two major bands at 28 - 31 and 62 - 66 kDa correspond to the two urease subunits, while the 54 kDa hspB heat shock protein homolog (Suerbaum *et al.*, 1994) is probably represented by the band at approximately 55 kDa. Other variable proteins around 40 – 53 kDa may include flagellin, catalase and other unidentified proteins. The variations in size observed between different studies can be affected by the type of gel used, for

example, the urease subunit migrates at 66 or 62 kDa depending on whether a gradient or non gradient gel is used (Dunn, 1993).

Protein profiles show apparent consistency among *H. pylori* strains, but the individual's humoral immune response to these antigens can vary widely. In addition, several important antigens of *H. pylori*, detected by immunoblot techniques, are not recognised as major proteins in gels in which proteins are detected by Coomassie blue or Silver stain (Dunn, 1993). This study demonstrated that human serum contained antibodies that reacted with a number of antigens from our clinical strain. Consistent with others, the results showed that a large number of immunreactive proteins were detected by patient sera from the *H. pylori* infected individual. Western blot assays detecting IgG antibodies to *H. pylori* antigens have consistently reported up to 13 immunodominant bands ranging from 15 to 150 kDa, in addition to a large number of minor bands (Drouet *et al.*, 1991; Andersen and Espersen, 1992). Some of these immunodominant proteins have since been identified (Macchia *et al.*, 1993; Crabtree *et al.*, 1991; Cover *et al.*, 1990; Dunn *et al.*, 1992), but many remain uncharacterised.

Sera from infected patients tested by Western blotting generally reveal 1-15 bands while sera from non-infected patients react with 0-5 bands (Andersen and Espersen, 1992; Aucher *et al.,* 1998). This is also observed in the serological response to *Helicobacter* sp. in dogs, in which samples from uninfected dogs had fewer immunoreactive bands (5-12 median=8) than samples from infected dogs (12-18 median=16) (Strauss-Ayali *et al.*, 1999). Further characterisation of the serological response to *Helicobacter* antigens revealed that sera from positive patients gave a strong antibody response to the three main clusters of proteins encompassing the low, medium and high MMPs (Nilsson *et al.*, 1997), whereas sera from *H. pylori* negative patients reacted mostly with only the MMMPs. The immunoblot result for the negative patient sera in this study was consistent with this and probably reflects the finding that these medium-sized proteins, in the range 43 – 66 kDa, are responsible for cross-reactivity with other antigens such as flagellin and heat shock proteins from other bacterial species (Nilsson *et al.*, 1997).

Using Southern blotting, the *cag*A and *vac*A genes in were detected in the *H. pylori* clinical strain used for the library constructions described in this thesis. Compared to the type strain (*H. pylori* 11637), the clinical isolate demonstrated more than one band

on Southern blot and is most likely due to an additional *Hin*d III site in the sequence. Differences in restriction-fragment sizes and in probe hybridisation patterns is common among *H. pylori* isolates, and has been interpreted to mean that *H. pylori* strains are highly diverse in their genomic organisation and gene order (Jiang *et al.*, 1996). Any two independent clinical isolates are readily distinguishable by PCR-based random amplified polymorphic DNA (RAPD) fingerprinting (Bukanov and Berg, 1994). Comparisons of the two published genomes (Tomb *et al.*, 1997; Alm *et al.*, 1999) revealed that the overall genomic organisation was in fact quite similar, and that differences observed in sizes of *Not* I fragments between the two strains was mainly due to silent nucleotide variation within genes (Alm *et al.*, 1999).

The diversity at the genome level does not appear to affect the organism's ability to colonise the host, but can have important phenotypic consequences. All *H. pylori* isolates contain a *vac*A homolog, but only about 50% of isolates produce detectable vacuolating activity. This is due to allelic mosaicism in the *H. pylori vac*A gene such that each gene has one of two different signal sequences (s1(s1a,s1b) and s2) and also one of two alternative sequences in the middle region of the gene (m1 and m2). The significance of this is that s1 m1 VacA is highly active, while s2 m2 VacA is inactive. Western blotting confirmed that the cytotoxin was being produced by both the clinical strain NZCH1 and the type strain used in this study. *H. pylori* strains producing cytotoxin activity have been found to be more closely associated with more severe clinical disease (Van Doorn *et al.,* 1998), and this association is reflected in VacA allele variety (Atherton *et al.*, 1997). For these reasons, the VacA+CagA+ phenotype of the *H. pylori* strain for construction of the library was important, since type I strains are perhaps more likely to contain genes that encode products involved in the immune response to infection.

Western blotting also confirmed the production of the CagA protein, indicating that this strain contained the cag PAI, also associated with more severe disease (Jenks *et al.*, 1998). The difference in size observed in the CagA protein between the two *H. pylori* strains used in this study also reflects variability at the genetic level. Using SDS-PAGE, the CagA protein band varies between 120 and 140 kDa, probably due to a variable region that exists within the *cag*A gene. Repetitions of 15- to 99- base nucleotide sequences and variation in the number of codons result in CagA proteins

which vary in both size and amino acid sequence (Rudi *et al.*, 1998). It is currently unclear whether these differences effect the clinical outcome of *H. pylori* infection, but it has been suggested that the size variations observed may affect antigenic properties of CagA and thereby alter the host's immune system's ability to recognise the antigen (Rudi *et al.*, 1998). Indeed, as discussed above, the serological profiles of *H. pylori*-positive patients tend to be quite diverse, possibly reflecting the genetic diversity of *H. pylori* worldwide. The polymorphism of the antibody response to *H. pylori* has been suspected to reflect either an evolution of the immune response or an antigenic shift of the infecting strain, which is also suspected to be correlated to a predisposition for more severe disease (Aucher *et al.*, 1998).

The *H. pylori* expression library constructed in this study formed the basis of a collaborative project designed to identify the gene encoding an outer membrane vesicle antigen originally described by Keenan *et al.*, (1998), by immunoscreening with a monoclonal antibody raised to this protein. The monoclonal antibody used for this screening the library was shown to map to one region of the genome. Further experiments confirmed that the gene encoding a conserved 20 kDa lipoprotein (lpp20) (Kostryznska *et al.*, 1994) was the antigen detected by the antibody. Lipoproteins are major antigens in a number of bacterial pathogens, and Lpp20 has been shown to be an immunodominant *H. pylori* antigen (Cao *et al.*, 1998; Kostryznska *et al.*, 1994; Hocking *et al.*, 1999). Moreover, it is likely that the immunoreactive species-specific 19 kDa outer membrane protein in an earlier study is Lpp20 (Drouet *et al.*, 1991). In addition, the report by Kostryznska *et al.* (1994) describes the purification of this protein from outer membrane vesicles spontaneously released during growth, also described in the study by Keenan *et al.*, (1998). Antibodies to this antigen were not detected in patient sera, however, leading the authors to conclude that the protein was either nonimmunogenic or nonantigenically cross-reactive. In addition, the Lpp20 antigen appears to be conserved and does not cross react with closely related species of *Helicobacter*, *Campylobacter* or a diverse range of other bacteria (Drouet *et al.*, 1991; Kostryznska *et al.*, 1994).

Extracellular membranous vesicles are thought to represent an important mechanism for the release and delivery of virulence factors and antigens to host tissues. Budding of outer membrane vesicles of *H. pylori* has recently been proposed as a specific secretion

pathway to deliver antigens to the gastric mucosa (Fiocca *et al.*, 1999) and investigation of other components of *H. pylori* OMVs, in addition to lpp20, may be worthwhile. Recently this antigen has been shown to protect against *H. pylori* infection in a murine model along with a number of other surface or secreted proteins (Hocking *et al.*, 1999). It is possible that other components of *H. pylori* outer membrane vesicles and exported proteins may be useful for inclusion in a subunit vaccine against *H. pylori* infection.

Chapter 4 Identification of *H. pylori* DNA Sequences Encoding Exported Proteins

4.1 Abstract

Bacterial secreted or membrane-associated proteins often play a role in the pathogenesis of infection and are potentially useful candidates for vaccine development. The objective of this study was to identify, using a genetic approach, proteins of *H. pylori* that are exported across the cytoplasmic membrane. A genomic *H. pylori* library was constructed in the plasmid vector pJEM11, designed to identify exported proteins by use of alkaline phosphatase (AP) gene fusions. The pJEM11 *H. pylori* library was transformed into *E. coli* and recombinant clones expressing enzymatically active AP (AP+) were identified as blue colonies when plated on the colorimetric substrate 5-bromo-chloro-indoyl-phosphate (BCIP). Approximately 2.3% of transformants contained insert *H. pylori* DNA sequences that expressed active AP. Two hundred and eighty of these were selected and stored for further analysis. Sequence analysis of twenty-eight *E. coli* pJEM11 *H. pylori* inserts confirmed that the majority contained partial putative *H. pylori* genes encoding exported proteins (secreted or membrane proteins) in frame with AP. In other experiments, lysate proteins prepared from a number of *E. coli* AP+ clones were screened in Western blots using a polyclonal antibody raised against alkaline phosphatase. The antibody was able to detect each fusion protein as well as the AP positive control. No AP activity was detected in protein prepared from a white (AP-) clone, a non-recombinant pJEM11 clone and a non-transformed *E. coli* control. The use of the pJEM11 vector and AP fusion technology proved to be a fast and reliable method to identify genes encoding novel exported proteins from *H. pylori* .

4.2 Introduction

The interaction of bacterial pathogens with host cells often involves factors that are located on the bacterial surface or proteins that are secreted into the extracellular environment (Hueck, 1998). Bacterial surfaces are thus rich sources of immunodominant proteins, which represent potential vaccine antigens (Vyas and Sihorker, 1999). Indeed, outer membrane preparations of *H. felis* were shown to

contain proteins capable of inducing protection against *H. felis* infection (Keenan *et al.*, 1997) (see also Chapter 3). However, only a small number of *H. pylori* proteins have been experimentally determined to be unambiguously localised within the periplasm or outer membrane of *H. pylori*. This is partly due to the presence of high copy assemblies of cytoplasmic proteins such as urease and GroEL, which copurify with cell envelopes and complicate analysis of the membrane components (Cao *et al.*, 1998; Vanet and Labigne, 1998). Some well-characterised exported proteins include the vacuolating cytotoxin (VacA) (Cover, 1996), flagellin components (Suerbaum *et al.*, 1993), adhesins (Wadstrom *et al.*, 1996) and proteins associated with the cag pathogencity island (Covacci *et al.*, 1997). There are many immunogenic surface-located or secreted *H. pylori* products that have yet to be identified. Progress in this field has been hampered by the difficulties associated with screening large numbers of proteins and assessing their role in the immune response to infection. One potential strategy to overcome these difficulties is to first identify the genes that code for these exported proteins, and then determine if they are recognised by the host's immune cells .

Approximately 20% of the polypeptides synthesized by bacteria are located partially or completely outside of the cytoplasm (Pugsley, 1993). Most reach their final destination via the general secretory pathway (GSP), the first step of which is their insertion into and translocation across the cytoplasmic membrane. *Escherichia coli* alkaline phosphatase (encoded by the *pho*A gene) is an enzyme that normally resides in the periplasm and is exported by the GSP. The enzyme is synthesized as a precursor monomer with a signal peptide at the amino-terminal end. If retained in the cytoplasm, AP is enzymatically inactive. This inactivity is due to the inability of the intramolecular disulfide bonds to be formed in the cytoplasm. Disulfide bond formation in *E. coli* occurs during or following translocation of AP across the cytoplasmic membrane where Dsb proteins facilitate bond formation (Derman and Beckwith, 1995). Using this feature as a marker of export, alkaline phosphatase (AP) fusion systems have become a powerful tool for the analysis of protein secretion (Manoil *et al.*, 1990). The colorimetric detection of AP with the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) makes it useful in gene fusion reporter systems.

Several genetic methodologies based on AP fusions have been developed for characterising proteins exported by pathogenic bacteria. Tn*pho*A is a transposon that

can be used to directly select for transposon inserts in genes coding for secreted proteins (Manoil and Beckwith, 1985). Tn*pho*A mutagenesis has been used to identify virulence factors from several pathogens (San Mateo *et al.*, 1998) including *Salmonella typhimurium* (Finlay *et al.*, 1988), *Vibrio cholera* (Taylor *et al.*, 1987) and enterotoxigenic *E. coli* (Donnenberg *et al.*, 1990). More recently, a similar system of reporter gene technology based on Tn*Bla*M transposon mutagenesis using β-lactamase as the reporter gene was used to identify gene loci that are involved in virulence-associated traits such as motility and adherence in *H. pylori* (Odenbreit *et al.*, 1996). Gene fusion methodology based on AP (Hoffman and Wright, 1985) has been used in our laboratory (Johnson *et al.*, 1995) and by others (Bina *et al.*, 1997) to successfully identify *H. pylori* exported proteins.

This chapter describes the first genetic approach for the identification of *H. pylori* genes encoding exported proteins using *pho*A gene fusion technology. An *H. pylori* gene library was constructed in the plasmid vector pJEM11 (Figure 4.1). This vector utilises the features of AP by fusing fragments of *H. pylori* DNA upstream of the *E. coli pho*A

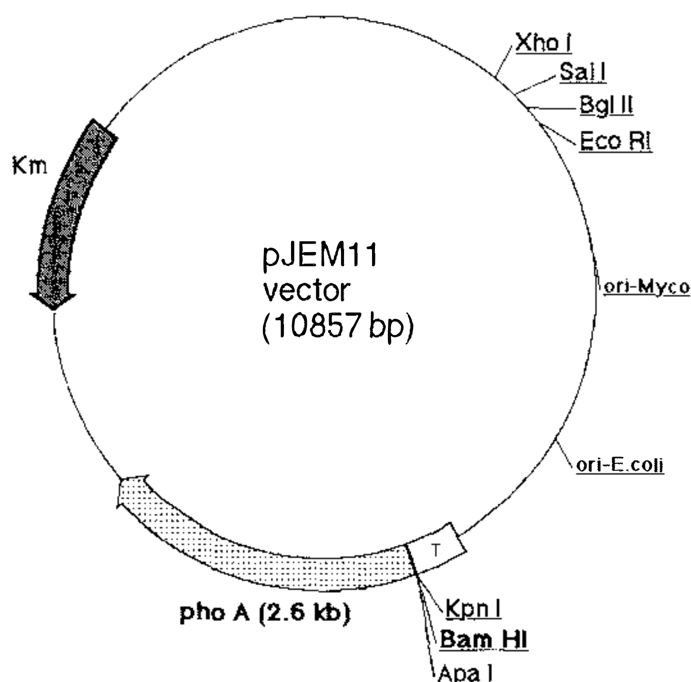


Figure 4.1: Map of the plasmid vector pJEM11 showing the major features. pJEM11 is a shuttle vector containing the origins of replication (ori) for both *E. coli* and Mycobacteria. The selectable marker is the kanamycin gene (Km). The truncated *E. coli* alkaline phosphatase gene is devoid of the promoter, start codon, and signal sequence.

gene. The *pho*A gene promoter, ribosomal binding site and the complete signal-sequence encoding region including the translational start codon of the *pho*A gene have been removed in pJEM11. Therefore, expression and exportation of AP depends on a translational fusion with the N-terminus of another exported protein, present in the correct reading frame with the AP moiety (Lim *et al.*, 1995). The pJEM11 *H. pylori* library was transformed into *E. coli* as the host strain. Recombinant plasmids expressing enzymatically active AP were sequenced to identify open reading frames (ORFs) encoding putative exported proteins. Results obtained in the preliminary screening of this *H. pylori* exported protein library indicate that it is a powerful tool to selectively identify *H. pylori* secreted or membrane-associated proteins.

4.3 Materials and Methods

4.3.1 Construction of an *H. pylori* exported protein library

4.3.1.1 Preparation of pJEM11 plasmid DNA

E. coli DH10B cells were transformed with the plasmid vector, pJEM11. Large scale purification of pJEM11 was carried out by cesium chloride / ethidium bromide equilibrium centrifugation, based on a method described in Current Protocols In Molecular Biology (Heilig *et al.*, 1994). Cultures of *E. coli* -pJEM11 were grown overnight in LB medium (150 ml) containing 30 μg/ml kanamycin. The cells were pelleted by centrifugation at 5,000 rpm for 20 mins at 4°C in a GSA rotor (Sorvall RC5 Centrifuge, Du Pont). Following washes with TE buffer, the cells were resuspended in 12 ml of 25% sucrose, 50 mM Tris, 40 mM EDTA (pH 8.0) supplemented with 1.25 mg/ml lysozyme and 1.5 μg/ml RNAse and incubated on ice for 15 mins. A 7.5 ml volume of 250 mM EDTA (pH 8.0) was added and the cells were incubated on ice for 15 mins. The cells were lysed by addition of 2.5 ml of 5% Triton X-100, 50 mM Tris, 0.2 M EDTA (pH 8.0) and left on ice for a further 45 mins. Insoluble cellular debris was pelleted by centrifugation (Sorvall SS34 rotor, 15,000 rpm for 90 mins at 4°C) and the supernatant incubated at 65°C for 15 mins followed by centrifugation at 7,000 rpm for 15 mins at 4°C. The supernatants were collected and 1/3 volume of 40% polyethylene glycol / 2 M NaCl added to precipitate the DNA. The DNA was pelleted, resuspended in 15 ml TE buffer, and stored at 4°C overnight. After warming the DNA

solution to RT, 12.5 g of CsCl was added, with stirring. A blank solution of 1.2 g/ml CsCl in TE was made to top up the DNA solution to the neck of the ultra-centrifuge tube (Nalge Nunc International, New York). Ethidium bromide was added to each tube at a concentration of 0.67 mg/ml. Equilibrium centrifugation was carried out using a Type Ti70 fixed angle in a Beckman L8-70 Ultracentrifuge at 62,000 rpm for 22 hrs 15 mins. The speed of the ultracentrifuge was reduced to 40,000 rpm one hour before stopping. Under UV illumination, the ultracentrifuge tube was punctured with a 21 gauge needle just below the concentrated plasmid DNA band and the plasmid DNA collected. The plasmid DNA was washed with an equal volume of TE-saturated isopropanol, and the isopropanol layer containing the ethidium bromide discarded. After four washes, 3 ml TE buffer and 4 ml isopropanol were added to 1 ml of plasmid DNA and the sample frozen at -20°C for 75 mins. The DNA was pelleted at 14,000 rpm for 20 mins at 4°C in a microfuge (Centrifuge 5402, Eppendorf). The DNA pellet was washed twice with 70% ethanol and resuspended in 200 µl TE buffer.

4.3.1.2 Preparation of the *H. pylori* pJEM11 library

For construction of the library, approximately 1 µg of CsCl-purified pJEM11 vector was linearised with the restriction enzyme *Bam* HI and dephosphorylated using *E. coli* alkaline phosphatase (Pharmacia Biotech, Sweden) according to the manufacturer's instructions. Genomic DNA was isolated from *H. pylori* NZCH1 and partially digested with *Sau* 3A (~1U per 2.5 µg DNA) for 2 mins at 37°C. Following electrophoresis, DNA fragments between 200 and 2000 base pairs were excised from a 1% low melting point agarose gel and purified using GELase (Epicentre Technologies, Wisconsin) according to the manufacturer's instructions. The *H. pylori* DNA fragments were ligated into the compatible *Bam* HI site of pJEM11 with T4 DNA ligase for 16 hrs at 4°C. Following the ligation, 2 µl of the dialysed ligation mix was transformed into electrocompetent *E. coli* DH10B cells (Life Technologies). The transformation mix (1 ml) was then plated onto four LB media plates containing kanamycin and BCIP and incubated overnight at 37°C. Colonies from these four plates were harvested into 4 ml of TE buffer and the total plasmid DNA extracted using alkaline lysis method (see Chapter 2.3) and stored at –20°C.

4.3.2 Screening of the pJEM11 library for known *H. pylori* gene sequences

4.3.2.1 Polymerase chain reaction

Oligonucleotide primers were designed to amplify two *H. pylori* genes encoding previously identified proteins whose sequences were available in the databases. These genes were the vacuolating cyotototoxin (*vac*A) and a flagellin subunit (*fla*A) for which the details of the primer sequences and amplification conditions have been previously described (see Chapter 3.3.3). PCR amplification was performed under standard conditions using Taq polymerase and an annealing temperature of 55°C (Chapter 2.6). PCR products were purified from agarose gels using Bresatec gel purification kit (BRESATEC, Aus).

4.3.2.2 Southern blotting

Colony hybridisation was carried out using nylon membrane discs (Hybond-N+ Nucleic Acid Transfer Membrane Discs, 0.45 μm removal rating, Amersham, England). Recombinant blue *E. coli* colonies were patched from glycerol stocks onto LB agar plates containing 30 μg/ml kanamycin. Following overnight incubation at 37°C, nylon membrane discs were placed on top of the agar for 1 minute and the *E. coli* recombinants transferred to the membrane were lysed by floating the discs on a solution containing 0.5 M NaOH and 1.5 M NaCl. Membrane discs were then neutralised in 1 M Tris, 1.5 M NaCl, pH 7.5 and the DNA fixed by placing the discs in a microwave oven for 1 minute. The purified PCR products generated as described above (section 4.3.2.1) were labelled with ^{32}P[dCTP] and the membranes screened with each radiolabelled probe as previously described for Southern blots (Chapter 2.8.2).

4.3.3 Analysis of *H. pylori* alkaline phosphatase fusions

4.3.3.1 Sequencing of the *H. pylori* DNA inserts

The DNA inserts from the pJEM11 plasmid constructs were partially or completely sequenced using the Perkin Elmer AmpliCycle™ Sequencing Kit according to the manufacturer's instructions, using [α-^{33}P]-dCTP as described in Chapter 2.7.1. The different oligonucleotide primers trialed in the optimisation of the sequencing protocol are listed in Table 4.1.

Table 4.1: Oligonucleotide primers designed to the pJEM11 vector

Sequencing Primer	Primer Sequence (5'-3') (reverse primer) (forward priner)	Reference
pJEM3*	TCG CCC TGA GCA GCC CGG TT	Lim *et al.* (1995)
pJEM4#	TTG GGG ACC CTA GAG GTC CC	Lim *et al.* (1995)
pJEM6*	GCA GTA ATA TCG CCC TGA GCA GC	This study
pJEM7#	TTA ATT GGG GAC CCT AGA GGT CC	This study
JEM2*	TCGCCCTGAGCAGCCCGGTT	Pasteur Institute
JEM1#	CGAGCTGCAGTGGGATGACC	Pasteur Institute

* - sequencing primer designed to the complimentary sequence of the *E. coli pho*A gene coding for alkaline phosphatase (Acc#01659).
# sequencing primer designed to the coding strand of the transcriptional terminator sequence from pJEM11. The sequence is derived from the bacteriophage T4 gene (Acc#X06619).

Both published and custom designed primers were tested in the cycle sequencing reaction. Oligonucleotide primers were designed to both the non-coding strand of the *phoA* gene and to the coding strand of the terminator sequence on the pJEM11 vector. Primers were designed as previously described and used at a final concentration of 10 μM in the cycling mix, along with approximately 2 μg of pJEM11 plasmid DNA. Sequencing contigs were built up using the GeneWorks Sequencing Project document. A loop structure in the region of the forward primer design was later described (M. Jackson, Mycobacterial Genetics Unit, Pasteur Institute, Paris; personal communication) and a new primer (JEM1) designed upstream of this region eliminated previous PCR and sequencing technical problems (see Figure 4.2). Primers JEM1 and JEM2 were later used to PCR amplify the pJEM11 inserts, which were then sequenced directly by direct automated sequencing (see Chapter 2.7.2).

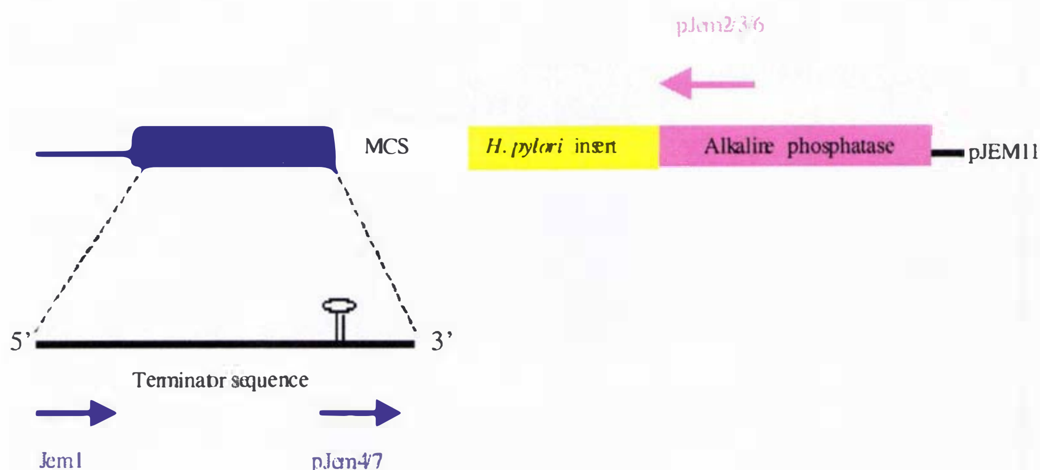


Figure 4.2: (a) Schematic representation of the location of the oligonucleotide primers designed to sequence the *H. pylori* insert within the pJEM11 vector. Primers were designed to the coding strand of the terminator sequence and the non-coding strand of phoA. Sequences of the individual primer pairs are listed in Table 4.1. (b) The sequence of the loop terminator region in pJEM11 showing the location of and sequence of the loop structure.

4.3.3.2 Western blot analysis of fusion proteins

In the Western blot experiments to detect the AP fusion proteins, lysate protein from recombinant *E. coli* was prepared as described previously (Chapter 2.9.1). Approximately 20 µg of lysate protein was separated on a 12% SDS-polyacrylamide gel and transferred onto PVDF membrane as previously described (Chapter 2.10.3). Following blocking, the membranes were incubated with rabbit anti-*E. coli* alkaline phosphatase IgG (Rockland, Gilbertsville, USA) diluted 1:20,000 at RT for 1 hour. After washing with PBS-T, membranes were incubated with goat anti-mouse IgG-alkaline phosphatase conjugate (Sigma, St Louis) diluted 1:2,500 at RT for 1 hr.

Protein bands detected by the antibody were visualised by the addition 50 μg / ml BCIP and 0.1% NBT as substrates.

4.4 Results

4.4.1 Construction and screening of an *H. pylori* library of AP fusions

In order to identify *H. pylori* gene sequences encoding exported proteins, a library of *H. pylori* AP-fusions was constructed in the vector, pJEM11 and expressed in *E. coli*. The design and construction of the plasmid vector has been described elsewhere (Lim *et al.*, 1995). The construction and screening of the library is diagrammatically represented in Figure 4.3. Plasmid DNA was purified from twenty randomly selected transformants

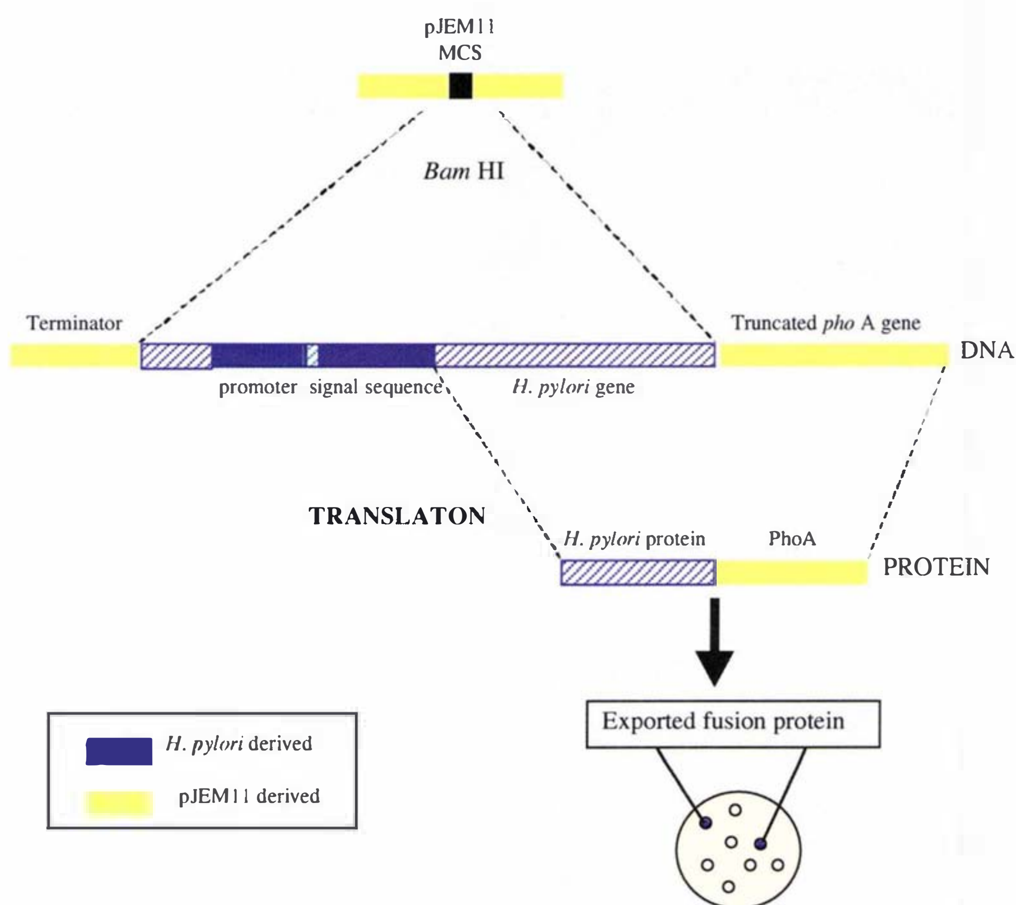


Figure 4.3: Schematic representation of the pJEM11 AP fusion library. *H. pylori Sau* 3A fragments were cloned into the *Bam* HI site of the pJEM11 vector. *H. pylori* sequences containing a promoter and signal sequence in frame with the *pho* A gene, and transcribed and translated by *E. coli,* will produce an AP fusion protein. The AP fusion protein, once exported across the cytoplasmic membrane, will turn the recombinant colony blue on the substrate BCIP.

and digested with *Pst* I and *Kpn* I to estimate the percentage of the library that contained DNA inserts. All of the plasmids were found to contain inserts and the DNA insert sizes ranged from 200 to 2000 base pairs (bp) with an average insert of 1000 bp (data not shown). The pJEM11 *H. pylori* library was screened for recombinants encoding exported AP fusion proteins by plating 12,000 of the *E. coli* transformants onto medium containing kanamycin and the chromogenic substrate BCIP. Recombinant clones producing enzymatically active AP turn blue in the presence of this substrate. Two hundred and eighty of these transformants (designated plasmids pJHp1-280) turned a detectable blue after overnight incubation, representing 2.3% of the total number of transformants (see Figure 4.4). Each of these blue recombinant colonies were selected and stored as glycerol stocks at –70°C for future analysis.

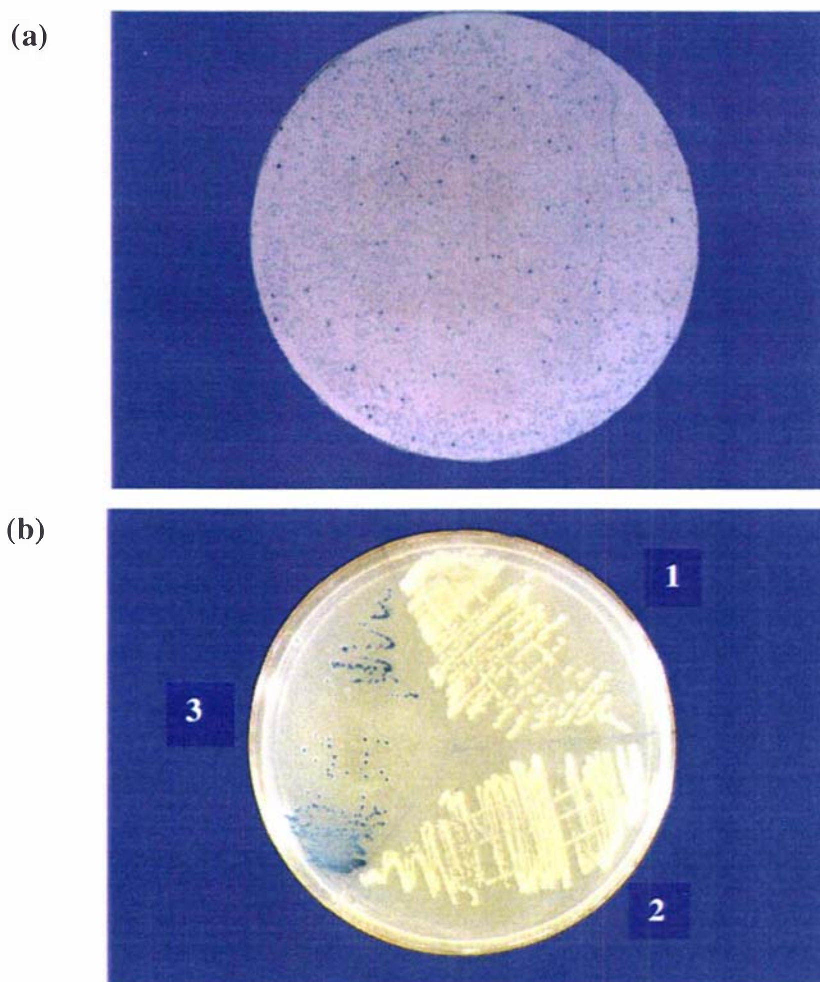


Figure 4.4: (a) Recombinant *E. coli* pJEM11 *H. pylori* recombinant colonies. AP+ clones turn blue in the presence of the colorimetric substrate, BCIP. (b)Single colony streaks of pJEM11 recombinant colonies. 1, pJEM11 no insert; 2, pJEM11 white recombinant clone; 3, pJEM11 blue recombinant clone.

4.4.2 Screening of the *H. pylori* AP fusion library for known *H. pylori* gene sequences

In other studies, all *H. pylori* strains have been found to contain a gene encoding the vacuolating cytotoxin (VacA), responsible for vacuolating of epithelial cells, and expressed by approximately 50% of *H. pylori* strains. The clinical strain used in this study was previously shown to be VacA+ (see Chapter 3.4.2) and so the presence of the gene in the exported protein library was investigated. Sequences of the flagellin subunit A gene (*fla*A) were also used as a probe to test for its presence in the library. A PCR product was amplified from *H. pylori* 17874 genomic DNA using the primers designed to the *vac*A and *fla*A genes, radiolabelled and used as a probe to screen the blue colonies. Two positive clones (pJHp234 and pJHp248) were identified using the *vac*A probe (see Figure 4.5) and one clone was positive for *fla*A (pJHp51). The recombinant

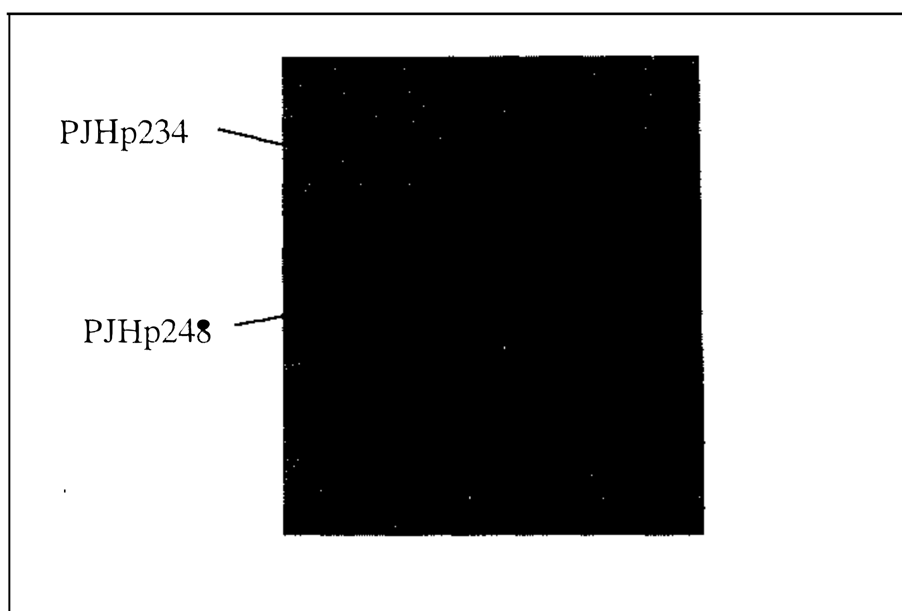


Figure 4.5: Autoradiograph of Southern blot screening of the pJEM1 1 *H. pylori* library with the *vac*A gene probe. Approximately 300 blue recombinant colonies were patched onto LB medium and transferred to membranes (one plate of fifty is presented in this figure). The *vac*A gene was radiolabelled and used to screen the library as described in the materials and methods (section 4.3.2). Plasmids pJHp234 and pJHp248 contained *H. pylori* insert DNA that hybridised to the *vac*A gene probe.

plasmids from the two *vac*A positive clones and the single *fla*A positive clone were isolated. Restriction endonuclease analysis showed the DNA insert sizes to be 1 180, 415 and 620 bp respectively (data not shown). Sequencing of these inserts revealed that each contained *H. pylori* sequence with homology to the known *vac*A or *fla*A *H. pylori* sequence. However, none of these sequences were in-frame with the *pho*A gene.

4.4.3 Sequencing of the *H. pylori* DNA inserts encoding exported proteins

PCR amplification of the pJEM11 inserts from thirty-six of these blue *E. coli* recombinants using primers JEM1 and JEM2, revealed that the insert size ranged from approximately 280 to 2200 bp in length (Figure 4.6) with a mean size of 767 bp. The DNA inserts from twenty- eight recombinant AP+ clones were sequenced to identify the ORFs responsible for expression of the *pho*A gene fusions. DNA and protein databases

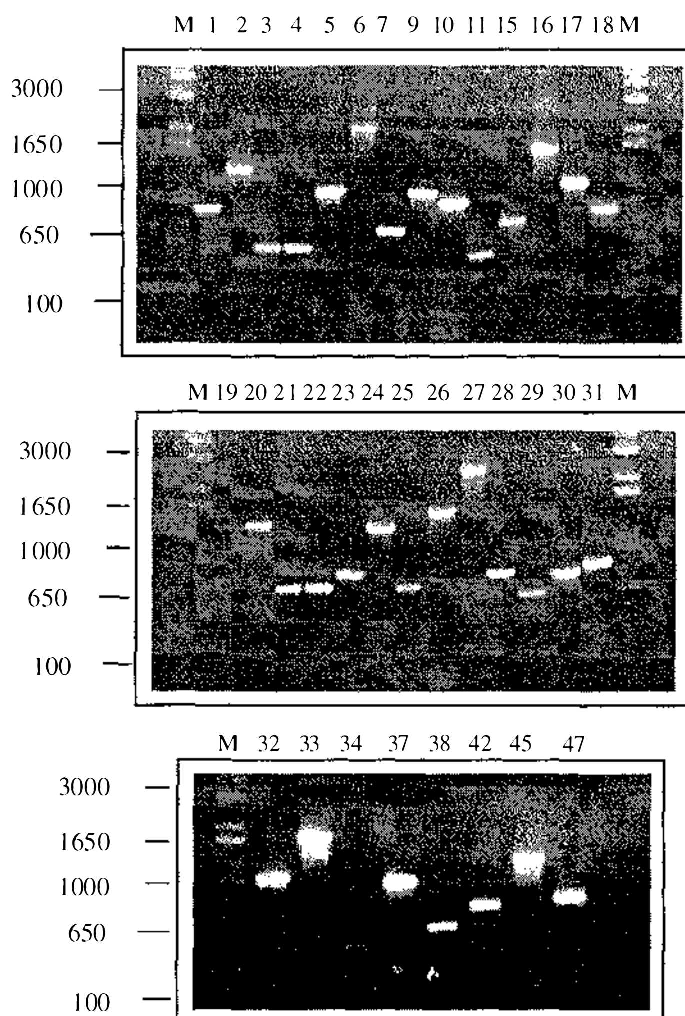


Figure 4.6: PCR amplification of thirty-five blue *E. coli* pJEM11 *H. pylori* recombinant plasmids. Primers JEM1 and JEM2 were used to amplify the *H. pylori* insert by PCR (see section 4.3.2.1). PCR products were separated on ethidium bromide stained 1% agarose gels and visualised under UV light. Insert sizes were deduced from a direct comparison with size markers labeled on the left.

were searched to identify similarities between the insert DNA and deduced amino acids sequence with sequences in the databases. Following the release of the *H. pylori* genome sequence (Tomb *et al.*, 1997), the insert sequences were re-submitted to the *H. pylori* database and the ORFs they encoded identified (see Tables 4.2 and 4.3). The results of these findings are presented in the following sections.

4.4.3.1 Identification of *H. pylori* ORFs with the start codon in frame with *pho* A

Twenty-three percent of the open reading frames (ORFs) identified in the *H. pylori* genome were predicted to contain a signal peptide (Tomb *et al.*, 1997). Table 4.2 lists the *H. pylori* ORFs identified that contained expression sequences and a start codon in-frame with the *pho* A gene. The majority of the ORFs identified encoded hypothetical secreted or membrane proteins of unknown function. Sequence analysis (Chapter 2.7.3) revealed that the majority of ORFs listed in Table 4.2 were predicted to encode inner membrane proteins (9 out of 16), while four encoded periplasmic proteins and two, outer membrane proteins. Three of the identified genes (HP0144, HP0375 and HP0758) were not previously assigned a signal peptide in the published genome sequence (Tomb *et al.*, 1997). Analyses of the predicted N-terminal amino acid (aa) sequence revealed features characteristic of bacterial signal sequences. The hydropathy plots of two of these proteins (HP0144 and HP0758) were compared to a typical integral membrane protein (HP1185) and are presented in Figure 4.7.

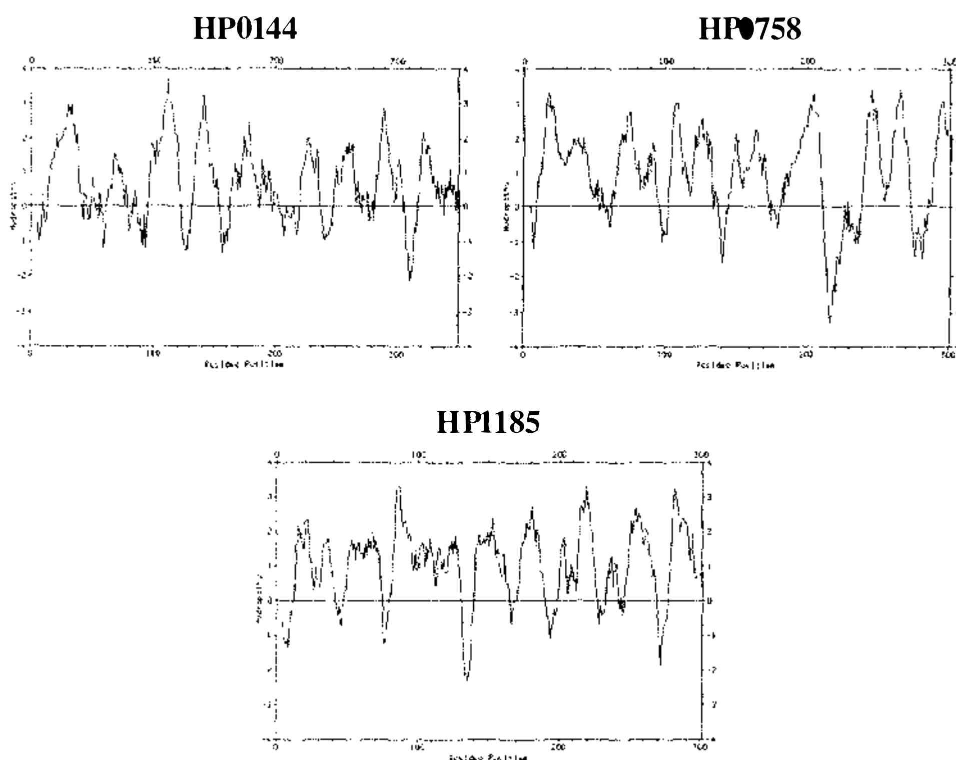


Figure 4.7: Kyte-Doolittle (KD) plot of three integral membrane proteins. The ORF was translated from the start codon and the hydropathy of the individual amino acids plotted using WEB ANGIS software. Hydrophobic amino acids lie above the centre line, while hydrophillic amino acids fall below.

Table 4.2: Open reading frames (ORFs) with start codon in-frame with *pho*A, identified by sequencing of pJEM11 *H. pylori* insert DNA from recombinant blue colonies.

HP/JHP #	PJEM11 clone #	Species	Homology %Id	Homology %Sim	Description (gene name)
0021*/0019	22	N/A			*H.pylori* specific hypothetical protein
0144/0132	27	*Rhizobium meliloti*	43.9	69.4	Cytochrome C oxidase subunit (fixN)
0247/0232	32, 109	*Haemophillus influenzae*	37.7	61.0	ATP-dependent RNA helicase (deaD)
0375/1006	42, 85	N/A			*H. pylori* specific hypothetical protein
0415*/0969	26	*Escherichia coli*	44.4	67.6	Conserved hypothetical integral membrane protein
0567*/0514	108	*Streptococcus pneumoniae*	25.4	50.3	Cell envelope protein
0726*/0663	16	N/A			Hypothetical protein (OMP)
0758/0695	37	*Haemophillus influenzae*	47.6	68.8	Conserved hypothetical integral membrane protein
0780*/0717	34	N/A			*H. pylori* specific hypothetical protein
0896*/1164	1, 47	*Helicobacter pylori*	100	100	Outer membrane protein (OMP19)(babB)
1085*/0340	20	N/A			*H. pylori* specific hypothetical protein
1117*/1045	3, 4	*Escherichia coli*	32.3	53.2	Conserve hypothetical secreted protein
1136*/1064	38	Synechocystis	28.3	57.2	ATP synthase FO, subunit b (atpF)
1185*/1111	2, 15, 30	*Haemophillus influenzae*	55.5	77.4	Conserved hypothetical integral membrane protein
1511*/1404	248	N/A			*H. pylori* specific hypothetical protein
1568*/1476	33	N/A			*H. pylori* specific hypothetical protein

#The ORFs are listed according to the nomenclature and descriptions designated in the published genome sequence [3]. * signal peptide designated to this gene product. N/A – not applicable (no significant homology was found to any other species in the database).

Each protein contains regions of hydrophobicity throughout the protein consistent with an integral membrane protein. Indeed, PSORT II predicted each to be inner membrane proteins with several potential transmembrane domains. Neither HP0144 or HP0726 appear to contain a peptidase cleavage recognition sequence in their predicted aa signal sequence, suggesting that the signal sequence in this instance is acting as a signal anchor. HP 0144 has 43.9% aa sequence homology (% identities) to the cytochrome c oxidase, heme b and copper-binding subunit (fixN) of *Rhizobium meliloti* which is membrane-bound, while HP0375 and HP0758 remain hypothetical proteins.

Figure 4.8 compares the hydropathy plots of a known secreted (or periplasmic) protein (HP1117) with a characterised outer membrane protein (HP0896). In comparison to the inner membrane proteins shown in Figure 4.7, the hydropathy plots of these proteins demonstrate the absence of the large stretches of hydrophobic segments characteristic of cytoplasmic membrane proteins. The sorting signals which discriminate between periplasmic and outer membrane proteins in gram-negative bacteria are not well characterised, but features of their amino acid sequence can reflect differences in the sorting process of these two proteins. The hydropathy plots of the *H. pylori* membrane or secreted proteins in Figures 4.7 and 4.8 illustrate the typical hydropathy plot observed in the N terminal region of an exported protein, and is representative of each of the *H. pylori* proteins listed in Table 4.2.

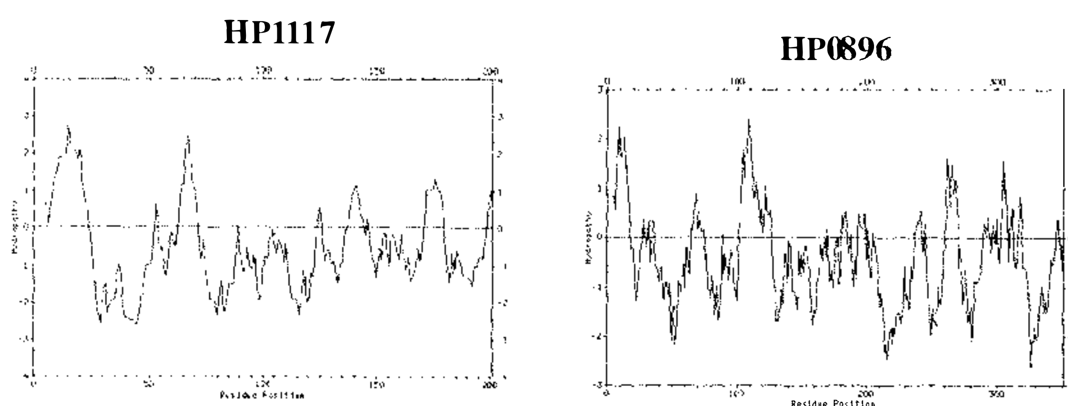


Figure 4.8: Kyte-Doolittle plot of HP1117, an *H. pylori* periplasmic protein, and HP0896, an outer membrane protein (omp19) identified by sequencing of the *H. pylori* inserts in recombinant blue pJEM11 clones. The ORF was translated from the ATG and hydropathy of the amino acids plotted using WEB ANGIS software. Hydrophobic amino acids lie above the centre line, while hydrophillic amino acids fall below.

4.4.3.2 *H. pylori* ORFs that encode for proteins lacking recognisable signal peptides or start codons in frame with the *pho* A gene

In addition to those *H. pylori* ORFs in-frame with the *pho*A gene, a number of unusual sequences were obtained for some blue recombinant clones. These are listed in Table 4.3 and have been divided into three groups. Group (a) lists those clones that contained

Table 4.3: Open reading frames (ORFs) with part of a gene fused to *pho*A, identified by sequencing of pJEM11 *H. pylori* insert DNA from recombinant blue colonies.

HP/JHP #	PJEM11 clone #	Species	Homology %Id	Homology %Sim	Description
(a)					
0214*/0200	5, 9	Homo Sapien	36.6	60.3	Sodium-dependent transporter (huNaDC-1)
0487*/0439	29	N/A			Hypothetical outer membrane protein
0910/0846	7, 21	*Haemophillus influenzae*	33.4	52.2	Adenine specific DNA methyltransferase (HINDIIM)
0946/0880	10, 28	*Haemophillus influenzae*	35.9	60.8	Conserved hypothetical integral membrane protein
1409/1301	18	N/A			*H. pylori* specific hypothetical protein
JHP0928	23, 31, 42	N/A			Putative outer membrane protein
(b)					
0243/0228	13	*Helicobacter pylori*	100	100	Neutrophil-activating protein (napA)
0805/0741	17	*Haemophillus influenzae*	36.9	53.4	Lipooligosaccharide biosynthesis protein (lex2B)
0823/0762	6	N/A			Conserved hypothetical protein
1579*/1486	25	N/A			*H. pylori* specific hypothetical protein
(c)					
1350*/1269	12	*Bartonella bacilliformis*	40.6	64.1	Carboxyl-terminal protease
1491*/1384	19, 24	*Haemophillus influenzae*	35.0	58.0	Putative phosphate permease

#The ORFs are listed according to the nomenclature and descriptions designated in the published genome sequence [3]. * signal peptide designated to this gene product N/A= not applicable (no significant homology to any species in the database).

part of an *H. pylori* gene fused to the *pho*A that did not include the start codon or N terminal region. These sequences generally included only the middle region of the ORF listed. Group (b) lists the ORFs that had their start codon and N terminal sequence in-frame with *pho*A, but included an intervening *sau* 3A fragment preceding the *pho*A gene. Although it is possible that there is transcriptional read-through of these intervening sequences, the addition of extra sequences could complicate the interpretation of these results. The third group, group (c), includes two *H. pylori* inserts that appeared to contain homology to proteins encoded on the non-coding strand relative to the *pho*A gene.

For each of the pJHp clones listed in Table 4.3 (a), the *H. pylori* insert sequence was analysed for the presence of any start codons in-frame with *pho*A preceded by possible promoter sequences. In all cases, at least one ATG start codon was found to be in-frame with the *pho*A gene and sequences resembling *E. coli* consensus promoter sequences were found upstream of the putative start codon. Sequences were also translated from these potential start codons to ensure read-through was possible to the *pho*A gene. In most cases, the translated sequence was identical to the protein sequence of the identified ORF. In addition, the translated sequences were assessed for regions of hydrophobicity that may be recognised as signal sequences (see Discussion).

The results obtained for each of these was similar and so the results of only one *H. pylori* insert (pJHp5 and pJHp9) in this category are presented. Plasmids pJHp5 and pJHp9 were found to contain identical insert sequences. Database searches revealed that the *H. pylori* insert sequence contained homology to HP0214, encoding a sodium-dependent transporter, however the homology was restricted to the region between nucleotides at position 343 to 1119 (see Figure 4.9). The promoter sequences and start codon of this ORF were not included in the insert and therefore not responsible for expression and export of AP. Several potential start codons (ATG) were found to be in-frame with the *pho*A gene and upstream of one of these was an *E. coli* consensus promoter sequence and ribosomal binding site. In addition, promoter prediction by neural network (Reese *et al.*, 1996) predicted a +1 of transcription site following the –10 and –35 consensus regions with a promoter score of 1.0 (Figure 4.9). A hydropathy plot was drawn from the amino acid sequence translated from the predicted start codon (Figure 4.10). The N-terminal region showed the characteristics of a signal peptide

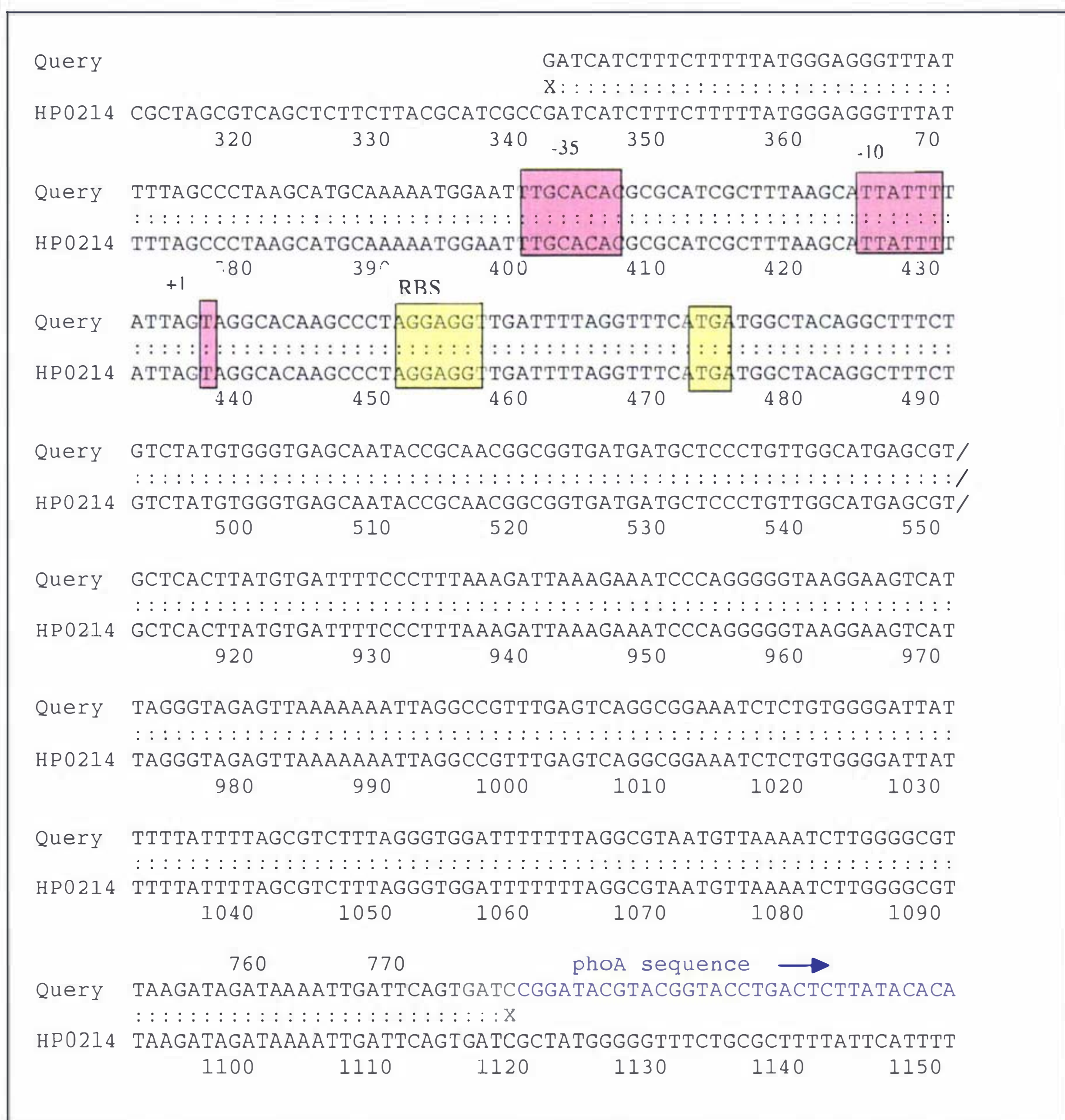
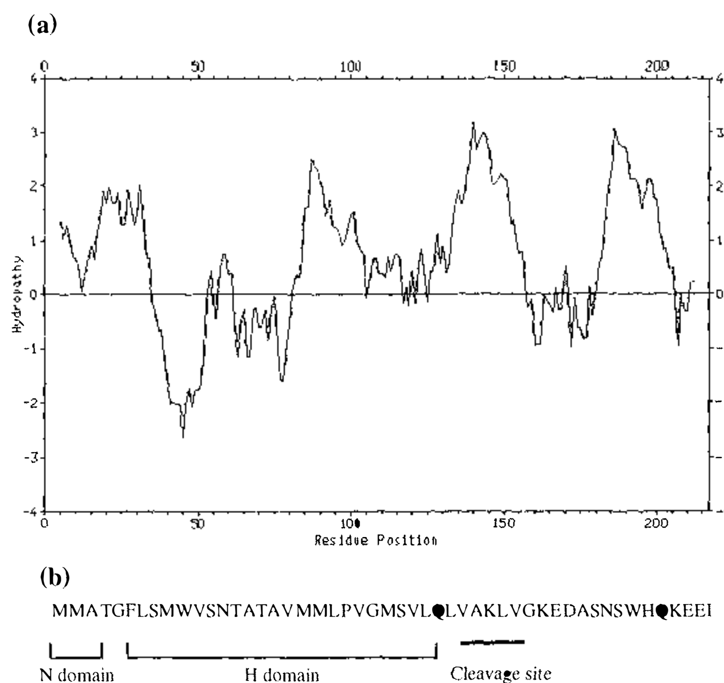


Figure 4.9: DNA sequence alignment between pJHp5/9 and the HP0214 open reading frame showing the region of homology. The *Sau* 3A sites (GATC) flanking the *H. pylori* insert in the pJEM11 vector are shown shaded in grey. The putative promoter sequences and start codon (ATG) are shown boxed along with the predicted +1 of transcription. The sequences flanking the fusion point to *pho*A and the following *pho*A sequence (blue) is shown. The pJHp insert sequence was 95.3% homologous over the 813 nt overlap (nucleotides 550-910 are not shown for diagrammatical presentation).

with a large stretch of hydrophobic amino acids and potential transmembrane domains, consistent with the report that this protein is an integral membrane protein.

(a)



(b)

MMATGFLSMWVSNTATAVMMLPVGMSVL●LVAKLVGKEDASNSWH●KEEI

Figure 4.10: (a) KD hydropathy plot of HP0214 translated from the potential start codon fused in-frame with the *pho*A gene. (b) The first fifty amino acids translated from the start codon, showing the potential domains and cleavage site that may substitute for a signal peptide.

4.4.3.3 *H. pylori* promoters are functional in *E. coli*

Sequencing of the pJEM11 *H. pylori* inserts revealed that a number of *H. pylori* ORFs expressing putative exported proteins could be identified on the basis of active alkaline phosphatase. Little data is available on the promoter sequences of *H. pylori.* With this in mind, the *H. pylori* ORFs expressing functional AP were investigated for the presence of consensus *E. coli* promoter regions. This was done both manually and with the aid of Promoter Predictions by Neural Network (Reese *et al.*, 1996). A representative group of these promoter sequences are presented in Figure 4.11. The predicted potential –10 and –35 promoter regions are shaded in grey, and the ribosomal binding domains (Shine-Dalgarno consensus) and the start codon (ATG or GTG) of each were labeled in red. The results demonstrated that a number of *H. pylori* genes contained promoter sequences that conformed to the consensus sequences of *E. coli* σ^{70} promoters. In one ORF (HP1117), the region upstream of the start codon in the pJEM11 vector was only long enough to include a potential –10 region, with a –35 region absent, indicating that this region may not be critical for functional transcription.

ORF#	SEQUENCE UPSTREAM OF THE PUTATIVE START CODON
0144 (1.0)	ATTAAGCTTTTTGAAAAAGATAAAATTTTATAATTAATGTTTATCTTTGTTGAATTTACTACAAATAGGAGTATTGCATG
0375 (0.94)	GCTTTTCATTGATTAAATCCATTGGTGCATGCTAAAATAAAACGATTAATAAAAAACAAATCGTTAGAAAGAAAGAAGTTAATG
0567 (0.99)	ATTATAAGTTAAAAGAGGTTTTTTATCCTTAAAAGAGCGTTTTTTAGCTAACATTTGATAATTTTTGGTTCGTTTAATGGGGATAATTTCATG
0758 (0.81)	AGGTGTAATTTTTTGCGCTAAACAAGCACAAAAATTCTATCATTTTTGCGCGGTATTTTCATTTTAACAAGGAGCAAAAATG
0780 (0.97)	GGGCCCCGGATCCCTTTTTTATTTAATTATGGCTGAGCGTTGAGAGAAAATAAAAAAGGAGAGTGATG
0896 (1.00)	GATCTTATTCTTTTAAAAGACTTATCTAACCATTTTAATTTCAAGGAGAAACATG
1085 (1.00)	TTTGGCATAAAACCCCCTTTTAGTTATAATATAGATTTTATTTTAGCTAAAAATGGTAGGGGTTTTAGCAAGGAATG
1117 (0.98)	TTTTAAGATTGGTAGCCATTGGCATTATGTTTGATCTTATTAAAGCAGAGTAACAAATG
1185 (0.91)	TTAAAATTTTGTCTTATAATTCAAGCTTTCAAATTGAATAAAATTTAACCCTTAAGATTTAAATG
1511 (0.91)	CTCTGAGCTTTATTTAAAAATCAGCAAGTTAGAAATTTCTCCCAATTCTCAAGTGGGAGCGAGCGTGAAAATCCGCTATG

Figure 4.11: Predicted putative promoter sequences for *H. pylori* ORFs identified in the pJEM11 library. The start codon for each ORF was fused in-frame with the *phoA* gene. The -10 and -35 *E. coli* consensus promoter regions and predicted +1 transcription are shaded in grey. The predicted ribosomal binding site (RBS) and start codon (ATG) are highlighted in red. The promoter and +1 transcription sites (underlined) were predicted using Neural Network Promoter Prediction (see text). The ORF number corresponds to that designated in the published genome (Tomb *et al.*, 1997) and the promoter score (0.1-1.0) is shown in brackets for each promoter sequence.

4.4.4 Detection of AP fusion proteins from recombinant *E.coli*

The molecular weights of the AP fusion proteins were determined by reacting lysate samples of recombinant *E. coli* with rabbit antibody raised to AP antigen. Lysate protein was prepared from twenty randomly selected *E. coli* AP+ transformants and a non-recombinant control from 40 ml cultures as described in the Materials and Methods (4.3.3.2). In each recombinant lysate sample, more than one band was observed on the Western blots (Figure 4.12). Commercially purified alkaline phosphatase was run on the gel as a positive control and had an apparent molecular mass of 54 kDa, A protein band of 54 kDa was also observed in the lysate preparations, in addition to higher molecular weight bands ranging from approximately 56 to 80 kDa. The predicted and observed molecular weights of a number of these were calculated and these compared favourably when the molecular weight of the highest band was taken as the observed molecular weight. The observation of multiple forms of each AP fusion suggests that they may have been post-translationally modified, possibly by proteolytic degradation. No protein bands were observed less than 54 kDa, suggesting that proteolysis appeared to be limited to the *H. pylori* encoded peptide.

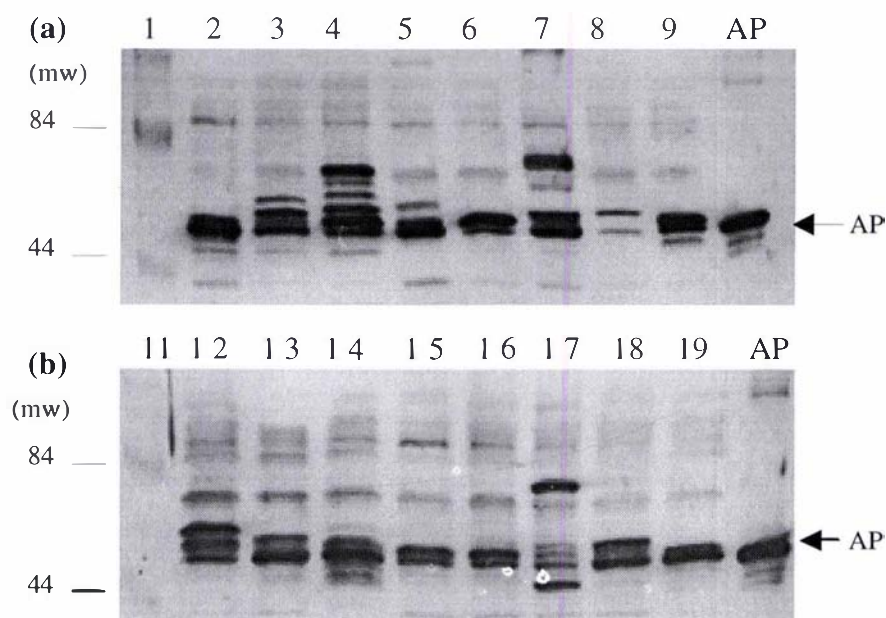


Figure 4.12: Western blot analysis of lysate protein from various blue recombinant pJEM11 *H. pylori* clones screened with a polyclonal antibody raised against alkaline phosphatase. Lysate proteins were separated on a 12.5% SDS-PAGE gel and transferred to PVDF membrane for Western blotting. Lanes 1 & 11; Kaleidoscope marker'; lane 2-9; pJHp33,pJHp9, pJHp37, pJHp42, pJHp50, pJHp32, pJHp5, pJHp51; lanes 12-19; pJHp45, pJHp4, pJHp35, pJHp2, pJHp34, pJHp46, pJHp43, pJHp49. AP = commercial alkaline phosphatase control.

4.5 Discussion

This study has described the identification of *H. pylori* ORFs that contain sequences in their N-terminal region that code for signals able to express and export alkaline phosphatase in an *E. coli* background. A number of sequences containing export signals identified in the pJEM11 library corresponded to the N-terminus of a ORF designated as a signal peptide in the published genome (Tomb *et al.*, 1997), while others appeared to contain random sequences capable of acting as signal peptides. Signal peptides appear to have highly variable structures with only a minimal amount of sequence conservation (Nielsen *et al.*, 1999; Edman *et al.*, 1999). This was dramatically demonstrated in two studies in which random DNA sequences were cloned in front of an SP-lacking β-lactamase gene in *E. coli* (Zhang and Broome-Smith., 1989) and a SP-lacking invertase gene in yeast (Kaiser *et al.*, 1987). In both cases, a large fraction (15-20%) of random sequences permitted export. These "functional" sequences were reminiscent of degenerate SPs, with an enrichment of positively over negatively charged residues and stretches of hydrophobic or uncharged residues. Their results supported the notion that there is no sequence specificity to the hydrophobic core of the signal sequence and that any hydrophobic sequence of the appropriate length can act as a signal (Schatz and Beckwith, 1990). In most cases, the SP was found to be noncleaved, most likely due to the more highly constrained pattern of amino acid normally present in the region surrounding the signal peptidase cleavage site.

The alkaline phosphatase fusion system used in this study also identified a number of middle hydrophobic regions of *H. pylori* proteins capable of exporting AP in *E. coli*. These were predicted or identified as being cytoplasmic membrane proteins. Polytopic cytoplasmic membrane proteins span the membrane at least twice via segments of mainly hydrophobic amino acids and each of these segments is potentially capable of targeting the nascent polypeptide to the cytoplasmic membrane and of initiating its insertion by interacting with membrane lipids in the same way as signal peptides (Pugsley, 1993). One model envisages polytopic membrane proteins being built up by the independent, sequential action of a series of signal sequences and stop transfer signals. Since transmembrane segments of polytopic membrane proteins are generally highly hydrophobic, it is not surprising that many of them can function as signal sequences when fused to a suitable reporter protein (Cheah *et al.*, 1994). The earlier

work on AP fusions provides evidence that alkaline phosphatase export can be promoted not only by signal sequences of periplasmic proteins but also by signal sequences within complex cytoplasmic membrane proteins (Manoil and Beckwith, 1985).

In Gram-negative bacteria, proteins can be exported to several different compartments or structures. The majority of ORFs identified in this study encoded putative inner membrane proteins. Hikita and Mitzushimu (1992) have extensively investigated the secretion efficiencies of a large number of signal peptide mutants of *E. coli* AP. Their data indicate the importance of threshold hydrophobicity and certain length limitations for the central, hydrophobic part of the signal peptide. Differences in amino acid sequence properties of the foreign protein can also affect secretion efficiences by influencing the ability of the signal peptide to fold into certain conformations, and/or to interact with various proteins during the secretion process. The initial results presented in this study indicate that SPs derived from cytoplasmic membrane proteins appear to be highly efficient at exporting AP. However, further sequencing of the pJEM11 clones may eliminate this bias by identifying more periplasmic or outer membrane proteins. Without doing localisation studies, the possibility that some outer membrane proteins have been incorrectly predicted as inner membrane proteins also exists.

The Western blot experiments using anti-alkaline phosphatase detected multiple bands which may represent breakdown products of the full-length fusion proteins. Attaching a foreign protein to alkaline phosphatase is likely to result in the accumulation of incorrectly folded AP or AP with an additional peptide sequence that may be more readily accessible to proteolytic cleavage. A number of studies have demonstrated that many secreted fusion proteins are highly sensitive to proteolytic degradation in the periplasmic space (Manoil and Beckwith, 1985; Hoffman and Wright, 1985). A fusion protein containing the N-terminal moiety from an inner membrane protein and a C-terminal derived from AP was shown to be proteolytically unstable and was degraded into a smaller product with the approximate molecular weight of the AP portion (Strauch and Beckwith, 1988). The size of the foreign protein attached to the reporter protein can also effect the amount of degradation in the periplasm (Guigueno *et al.*, 1997). Smaller *H. pylori* proteins fused to AP may lack regions containing amino acid

residues that are important for folding of the foreign protein into a proteolysis-resistant conformation.

In *E. coli*, over twenty different intracellular proteases have been identified (Waller and Sauer, 1996). However, relatively little is known about the biochemical or structural properties of proteases which may be responsible for degradation of misfolded or abnormal proteins in the periplasmic compartment of bacterial cells. Such a function has been attributed to the periplasmic serine proteases, DegP, which is also known as HtrA (or protease Do) and DegQ (Waller and Sauer, 1996). DegP is required for *E. coli* growth at elevated temperatures and is involved in the degradation of abnormal periplasmic proteins such as unfolded proteins which accumulate in the periplasmic space following heat shock or other stress conditions (Kolmar *et al.*, 1996). Mutations in the *deg*P gene result in decreased degradation of chimeric membrane and periplasmic proteins (Kolmar *et al.*, 1996). A degP mutant may therefore be useful in reducing breakdown of such fusion proteins in the periplasm of *E .coli.* Mutations in *deg*P have been shown to prevent the degradation of certain periplasmic fusion proteins and mutant forms of maltose-binding protein in addition to stabilising a number of other ordinarily unstable cell envelope proteins (Strauch and Beckwith, 1988; Baneynx and Georgiou, 1990). A particularly dramatic increase in expression of fusion proteins is also observed in strains *of E. coli* with mutations in *rpo*H (RNA polymerase sigma factor responsible for heat shock protein synthesis) and with *rpo*H *deg*P double mutants (Meerman and Georgiou 1994). The use of strains that are deficient in known cell envelope proteases such as DegP can therefore permit the selection of a suitable host based on reduced protease activity for these types of studies.

In addition to the necessary function of the signal sequence in directing export of AP in this system, the presence of a promoter region that is recognised by the *E. coli* transcription and translational machinery is also essential. Specific recognition of prokaryotic promoter sites by *E. coli* RNA polymerase is largely mediated by associated sigma factor 70 (σ^{70}). In many bacterial species, most promoter sequences are recognized by σ^{70} –like factors at two hexameric sequences centered around positions –10 and –35 from the transcriptional start site in *E. coli* (Kumar *et al.*, 1993). This study identified a number of potential *H. pylori* promoters with consensus sequences to

E. coli promoter regions. Sequencing of the *H. pylor*i genome has revealed a very restricted number of transcriptional regulators (Tomb *et al*., 1997). A recent study comparing the promoter sequences from eleven different *H. pylori* genes for which primer extension data was available identified a consensus –10 hexamer (TATaaT) which closely resembled the –10 consensus sequence in *E. coli* (TATAAT) (Forsyth *et al*., 1999). However, they were unable to identify an obvious –35 consensus sequence, leading them to believe that there may be structural differences between the *H. pylori* RpoD and orthologous proteins in other bacterial species. The highest degree of degeneracy found in domain 4 of *H. pylori* RpoD (encoding signal factor 80, σ^{80}) (Beier *et al*., 1998) is consistent with the presence of –35 promoter elements in *H. pylori* that are quite different from those found in *E. coli*. Alignment of the *vac*A gene from twelve different *H. pylori* strains also revealed a TAAAAA consensus sequence at –10 which matched the consensus *E. coli* –10 hexamer (TATAAT) at four of six positions. Alignment of the vacA –35 regions revealed a consensus sequence (TTTATG) that matched the *E. coli* consensus at 3 of 6 positions (Forsyth *et al*., 1999). This was consistent with the promoter regions investigated in this study where, according to the predicted +1 transcription, a –10 consensus region was present, while the –35 region was often more ambiguous (Figure 4.11).

Detailed nucleotide sequence analysis of the DNA region upstream of the transcription start points of the *H. pylori cag*A and *cag*B genes also revealed similarities to the –10 consensus sequence recognised by $E\sigma^{70}$, but with poorly conserved –35 regions (Spohn *et al*., 1997). Promoters with highly conserved –10 regions but poorly conserved or missing –35 regions have been reported for other pathogens such as *Mycobacteria* species (Bashyam *et al*., 1996). Interestingly, sequences upstream of the –10 region of one promoter (*cag*A P1) revealed the presence of a TGn motif. This motif has been described previously as an extended –10 region, necessary to promote transcription by $E\sigma^{70}$ (Kumar *et al*., 1993). Thus, it is possible that, in the promoters of *H. pylori* in the Cag PAI, these 'extended' –10 motifs compensate for the lack or poorly matching –35 region. Because all three promoters could be activated in *E. coli* and by $E\sigma^{70}$ *in vitro*, it indicates that their nucleotide sequences bear the information necessary for recogntion by the major RNA polymerases from both *H. pylori* and *E. coli*. This is consistent with the finding that a number of exported proteins identified in this study contained

sequences encoding expression sequences recognised by *E. coli* and presumably *H. pylori*. Beier *et al*., (1998) recently demonstrated that transcription in *H. pylori* is regulated by an RNA polymerase that shares functional similarities with the Eσ^{70} RNA polymerase from *E. coli*. An Eσ^{70}-like promoter also appears to regulate transcription of FlgR and other genes coding for structural components of the flagellar export apparatus (Spohn and Scarlato, 1999). Other studies have shown that a separate operon in *H. pylori* coding for both flagellin and house-keeping genes contains a promoter that shows similarities to the *E. coli* σ^{70} consensus (-10 TAAAAT, -35 TGGATA.) by primer extension analysis (Porwollik *et al*., 1999).

Therefore, *H. pylori* RpoD may utilise an extended –10 promoter, perhaps analogous to a set of *E. coli* σ^{70} promoters that contain a TGn motif immediately upstream of the –10 hexamer (Spohn *et al*., 1997 and Kumar *et al*., 1993) On examination of the predicted promoter sequences upstream of the ORFs identified in this study, no evidence was found of extended –10 promoters. However, *H. pylori* appears to use both extended –10 and –35 binding sites since mutations in the –14 position dramatically reduce transcription with the *cag*A P1 promoter and, despite a lack of –35 consensus, mutation of this region also resulted in decreased levels of transcript. The lack of conservation among *H. pylori* promoters outside the –10 hexamer in this study and others described above, suggests that there may be considerable variation in the avidity of RpoD binding to different promoters. This may represent a mechanism for determining the levels at which individual genes are constitutively transcribed by *H. pylori* (Forsyth *et al*., 1999).

A genetic approach using transposon shuttle mutagenesis, was recently used to identify adhesin genes by disruption of their function (Odenbreit *et al*., 1999). From a collection of *H. pylori* mutants in secreted proteins, two clones were isolated with reduced binding ability and both were found to encode integral outer membrane proteins (HP0912 (omp20) & HP0913 (omp21)). Interestingly, no consensus promoter sequences were identified upstream of the +1 transcription start site of the operon. The study also failed to express functional protein from these genes in *E. coli*, indicating that the inner membrane processing and/or outer membrane insertion of the proteins interfered with the survival of *E. coli*. A similar phenomenon has been found for expression of the recombinant *H. pylori vac*A gene (Schmitt and Haas, 1993). The amino acid sequence

of these proteins carried a C-terminal autotransporter motif, also identified in VacA. Thus, it may be possible that this type of transport mechanism can be lethal in a foreign, but heterologous host. Proteins containing N-terminal sequences processed across the outer membrane by this pathway may therefore not be detected using this system. In addition there are several other limitations to the presented strategy for identifying exported and secreted proteins. Only expressed and translocated proteins will be detected and this relies on a gene fusion occurring in a region containing a recognised signal and promoter sequences. In addition, AP fusions to significant stretches of hydrophobic amino acids that are present in cytoplasmic proteins can result in alkaline phosphatase activity. Spontaneous lysis of bacteria could also potentially generate false AP+ clones. However, experiments where *pho*A was fused to the *bla*F start codon without a signal sequence and grown in *Mycobacteria smegmatis* on BCIP medium produced colonies that remained white. Evidence that AP was being produced in the cytoplasm was demonstrated by Western blots, indicating that a AP+ phenotype is generally associated with a true exported protein (Lim *et al.*, 1995).

Despite these limitations, the results described in this study demonstrate that the *pho*A technology for genetic identification of exported proteins can be successfully adapted to *H. pylori* and that an *E coli* environment can allow expression of these genes. The biochemical purification of surface-associated proteins from *H. pylori* is difficult and time-consuming because of the strong tendency for autolysis of the bacteria. The use of gene-fusion methodology allows for the simple and rapid identification of genes encoding for surface-associated proteins, without the complications associated with biochemical purification methods and has allowed the identification of a number of exported proteins that can now be assessed for their role in the host response to *H. pylori* infection.

Chapter 5 Biochemical and Immunological Analysis of *E.coli* Alkaline Phosphatase Fusion Proteins

5.1 Abstract

An *H. pylori* genomic library was constructed in the plasmid vector pJEM11 to create a pool of fusion proteins containing *H. pylori* gene sequences encoding exported proteins fused to alkaline phosphatase (AP). The subcellular location of these fusion proteins in *E. coli* was determined in order to facilitate purification of AP fusion proteins. Cultures of cells from twenty pJEM11 AP+ transformants were fractionated into intracellular, periplasmic space and extracellular protein fractions. Western blot analysis was carried out with the cellular fractions using commercial antibodies raised against alkaline phosphatase to detect the fusion proteins, and also with antibodies raised against β-galactosidase (β-gal) to monitor its distribution as an intracellular protein marker. In addition, the cell fractions were assayed for both AP and β-gal activity. These experiments demonstrated that the majority of AP activity was detected in the periplasmic space of the *E. coli* cell, indicating that the fusion proteins were being transported across the cytoplasmic membrane. Western blot analysis was carried out, using patient sera as probe, against ten partially purified periplasmic space AP fusion proteins. The initial screen identified one fusion protein (pJHp20) that was recognised by sera from *H. pylori* infected patients. In other experiments, the periplasmic space protein fractions were assayed for their ability to stimulate proliferation of peripheral blood mononuclear cells (PBMC) from *H. pylori* infected patients. None of the protein preparations stimulated proliferation of PBMC above background levels. The study presented in this chapter describes the identification of a novel exported protein involved in the humoral host response to *H. pylori* infection.

5.2 Introduction

In the previous chapter, the construction of a genomic library was described in which a pool of alkaline phosphatase (AP) fusion proteins was used to identify *H. pylori* gene sequences encoding proteins exported by the general secretory pathway (GSP). In Gram-negative bacteria, such as *E. coli*, proteins secreted via the GSP are located in the periplasmic space or as components of the inner or outer bacterial cell membrane

(Pugsley, 1993). In contrast, *H. pylori* secretes proteins to the extracellular mileu, where many function as virulence factors and/or targets for the host immune response (Mai *et al.*, 1991; Huang *et al.*, 1995; Munzenmaier *et al.*, 1997). Ultrastructural studies have shown that the *H. pylori* bacterial cell consists of a well-defined outer membrane separated from the bacterial body by a thin periplasmic space (Fiocca *et al.*, 1999). The mechanisms by which *H. pylori* releases or secretes its virulence factors through these compartments are not well understood. Active secretion (Vanet and Labigne, 1998) and bacterial autolysis (Phadnis *et al.*, 1996; Cao *et al.*, 1998) have been proposed to explain the extracellular release of cytoplasmic proteins such as heat-shock proteins and urease. The *H. pylori* cag pathogencity island may also encode a novel secretion system for delivery of bacterial constituents into host cells (Censini *et al.*, 1996). Sequencing analysis of the *H. pylori* genome (Tomb *et al.*, 1997), and of *H. pylori* gene sequences encoding export signals (Johnson *et al.*, 1995; Odenbreit *et al.*, 1996; Bina *et al.*, 1997) has revealed that *H. pylori* signal peptides are an average length of 25 amino acids and contain features consistent with other Gram-negative bacteria such as *E. coli* (Tomb *et al.*, 1997). The export signals from several well characterised *H. pylori* exported proteins (Yoshida *et al.*, 1999; Chevalier *et al.*, 1999) also suggests that *H. pylori* exports some proteins to subcellular locations in a manner similar to *E. coli*.

The question then arises as to the fate of *H. pylori* exported fusion proteins when expressed in a heterologous host such as *E. coli*. As the signal peptides are similar in length and the cleavage recognition site conserved (Tomb *et al.*, 1997), one might expect such fusion proteins containing a functional signal peptide to be exported across the *E. coli* plasma membrane and cleaved by signal peptidase. However, it is not easy to predict what will happen to these proteins once they are in the periplasmic space. One possibility is that they will be recognised as foreign proteins and will be degraded by host proteases (Manoil and Beckwith 1985; see also Chapter 4). Alternatively, if targeting information is present in the fusion protein then the protein may end up being translocated to the outer membrane. One of the advantages of using colorimetric screening of AP activity is that fusion proteins which are present and enzymatically active in the periplasmic space can be readily detected. Signal peptides from both periplasmic and outer membrane proteins fused to alkaline phosphatase and expressed in *E. coli* have been shown to be efficiently targeted to the periplasm (Hoffman and

Wright, 1985). However, it is not known if secretion into the periplasm occurs when AP is fused to export signals from a heterologous species such as *H. pylori*.

Another question which is raised by expression of fusion polypeptides in heterologous hosts is whether the structural and conformational integrity of the polypeptide will be conserved to the extent that epitopes, normally recognised in the native protein, can still act as potent targets for the host immune response. The identification and characterisation of individual *H. pylori* antigenic proteins may be important for the understanding of the mechanisms of infection and the host's immune response to the organism. In addition, this information might be useful for the design of new diagnostic tests.

The principal objectives of the work described in this chapter were to investigate the subcellular distribution of *H. pylori* fusion proteins expressed in *E. coli*. If predominantly located in the periplasmic space, it was hoped to take advantage of this distribution as a rapid step to partially purify the recombinant fusion protein. The periplasmic fractions, enriched for the *H. pylori* fusion protein, could then be assayed for their immune recognition using patient sera in Western blot assays and also in lymphocyte proliferation assays using peripheral blood mononuclear cells (PBMC's) isolated from *H. pylori* infected patients.

5.3 Materials and methods

5.3.1 Isolation of periplasmic space proteins

Recombinant plasmids were transformed into *E.coli* DE3 (BL21), a strain carrying an inducible *lacZ* gene. Plasmid DNA was isolated from *E. coli* DH10B AP+ clones and transformed into *E. coli* strain DE3 using standard procedures. The periplasmic proteins were purified by a procedure based on a method described by Neu and Heppel (1965). The cells were grown in M63 minimal media (10g $(NH_4)_2SO_4$, 68g KH_2PO_4, 2.5mg $FeSO_4.7H_2O$, pH 7) supplemented with 0.4% glucose, 1mM MgSO4 and 0.4% casamino acids. The cultures were grown to late-log phase (OD 0.7-0.9) and harvested by centrifugation. The supernatant was stored as the extracellular fraction and the cells were washed with 10 ml of 10 mM NaCl, then agitated gently in 10 ml of sucrose

solution (25% sucrose, 1.5 mM EDTA) at RT for 10 mins. After centrifugation, 5 ml of ice-cold water was added to shock the cells which were then incubated on ice for 10 mins. The preparation was filtered through a 0.2 μm membrane filter (Millipore Corporation, MA, USA) to collect the periplasmic fraction. The cells were then washed in 10 mM Tris-HCl pH 8, 1 mM EDTA and sonicated to release the intracellular protein after incubating on ice for 30 mins with 0.5 mg / ml of lysozyme. The periplasmic space (PS) protein was then concentrated using Centricon membrane filters with a 10 kDa cut-off (Amicon Division, Beverly, USA) to a final volume of approximately 0.5 ml according to the manufacturer's instructions. Protein concentrations in each fraction were determined using the BCA colorimetric micro assay kit (Pierce, Rockford).

5.3.2 Enzyme assays

5.3.2.1 Alkaline phoshatase assay

Alkaline phosphatase assays were carried out on protein extracts based on a method originally described by Brickman and Beckwith (1975). Total protein (100 μl aliquots) or protein fractions (extracellular, periplasmic or intracellular) were assayed in 900 μl of 1M Tris-HCl (pH 8.0) and 100 μl of 20 mM *p*-nitrophenylphosphate (Sigma, St. Louis). Tris-HCl was used as a blank. Enzyme reactions were carried out in triplicate at 37°C for 40 minutes unless otherwise stated. Assay reactions were stopped by the addition of 100 μl of 1 M K_2HPO_4, and the optical density was measured at 420 nm. As a control, 0.05 μg of *E. coli* alkaline phosphatase (Pharmacia Biotech, Uppsala, Sweden) was assayed along with the protein samples. The protein concentration of each sample was measured to allow calculation of the specific activity of each according to the following formula:

$$\text{Activity (units / min / ml)} = \frac{OD_{420} \times 10^3}{\text{Vol (mls)} \times \text{Time (mins)} \times OD_{600}}$$

5.3.2.2 Beta-galactosidase assay

The assay of β-galactosidase activity in protein samples was based on a method described by Miller (1972). Each sample was diluted as appropriate and assayed in

duplicate in 800 µl of distilled water with the addition of 200 µl of 5x Z buffer (100 mM $Na_2HPO_4.7H_2O$, 350 mM $NaH_2PO_4.H_20$, 10 mM KCl, 2 mM $MgSO_4$, pH 7). As a blank, 800 µl of water was used. After equilibration at 30°C for 5 mins, the reaction was started by the addition of 200 µl of o-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma, St. Louis), and the time recorded for sufficient yellow colour to appear. The reaction was stopped by adding 500 µl of 1M Na_2CO_3. One unit of β-galactosidase was calculated as the amount of enzyme which liberates 1 µmol of o-nitrophenol per min at 30°C, pH 7 under the above conditions. All assays were done in triplicate with an average variation of less than 10% between samples.

5.3.3 Western blotting

For detection of alkaline phosphatase in the cellular protein fractions, Western blot assays were performed using a polyclonal antibody raised against alkaline phosphatase as previously described (see Chapter 4.3.3.2) or using the chemiluminescence developing system as follows. Approximately 10 µg of protein was electrophoresed on a 10% SDS-PAGE gel and transferred to PVDF membrane using standard procedures (Chapter 2.10.2). Following incubation in the primary antibody, the membrane was incubated for 1 hour in a 1:2,000 dilution of anti-mouse - POD conjugate (Boehringer Mannheim) and developed by chemiluminescence. For detection of β-galactosidase, the Western blot procedure was as described above for chemiluminescence development, with the exception that the primary antibody was a monoclonal antibody against β-galactosidase (Promega #53710, Immunoscreening system) diluted 1:5,000.

5.3.4 Immunoassays

5.3.4.1 Western blotting

Cross-reactive antibodies against *E. coli* removed from patient serum using an affinity column as described in Chapter 2 (section 2.12). One hundred microlitres of serum from patients PS010, PS014, PS018, PS025 and PS027 (see Table 2.4 for patient details) was made up to 1 ml with Tris buffered saline (TBS) and applied to the column. The eluted fractions containing the purified serum, determined by reading the absorbance at 280 nm, were pooled and used as the primary antibody in the Western blot. Approximately 40 µg of periplasmic space protein from pJHp #4, 5, 6, 7, 9, 10,

13, 16, 19 and 20 was separated by SDS-PAGE alongside periplasmic protein from a pJEM11 control containing no *H. pylori* insert DNA. Protein was loaded onto a large 12% SDS-PAGE gel using BioRad ProteanTM II Slab Cell apparatus according to the manufacturer's instructions and run overnight at 10 mA. Protein was transferred to PVDF membrane as described (Chapter 2.10.2) and following blocking, incubated in the primary antibody overnight at 4°C. After washing, the membrane was incubated in anti-human IgG conjugated to alkaline phosphatase (Sigma) diluted 1:20,000 for 1 hour at RT and developed colorimetrically.

5.3.4.2 Lymphocyte proliferation assay

Blood samples were collected from patients (n=4) infected with *H. pylori* at the Wakefield Gastroenterology Centre, Wellington. Peripheral blood mononuclear cells (PBMCs) were separated from heparinised blood samples by density centrifugation over lymphoprep (density = 1.077 g/ml; Nycomed Pharma As., Oslo, Norway). The PBMCs were washed by centrifugation three times in PBS and resuspended at a concentration of 2×10^6 / ml in supplemented RPMI-10 culture medium (Gibco BRL, Auckland, New Zealand). The PBMCs were cultured in triplicate wells (2×10^5 cells/well) of 96-well flat-bottomed microtitre plates (Nunc, Roskilde) in the presence of 100 µl of RPMI-10, 100 µl phytohaemagglutinin (PHA) (10µg/ml) (Sigma, St Louis), *Staphylococcus aureus* (SA-1) or *H. pylori* protein preparations, in a total volume of 200 µl. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 3 -7 days (3 -5 days for PHA and 5 - 7 days for bacterial antigens). Proliferation was detected by adding 10 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)(Sigma, St Louis) to each well and incubating a further four hours at 37°C. MTT was then removed and 200 µl of dimethylsuphoxide added to each well. The absorbance at 570 nm was determined using an ELISA reader. Results were expressed as mean OD_{570} x 1000 and as stimulation indices (SI), which were determined by calculating the mean absorbance of replicate stimulated wells divided by the absorbance of non-stimulated wells (no antigen). All experimental samples were carried out in triplicate.

5.4 Results

5.4.1 Isolation of periplasmic space proteins enriched for *H. pylori* AP fusion proteins

To determine the subcellular distribution of *H. pylori* AP+ fusion proteins when synthesised in *E. coli,* the cellular fractions were purified from cultures of a panel of the AP+ transformants and screened in a Western blot using an antibody raised against AP (see Figure 5.1). With each fusion protein, a strong band was detected either at the

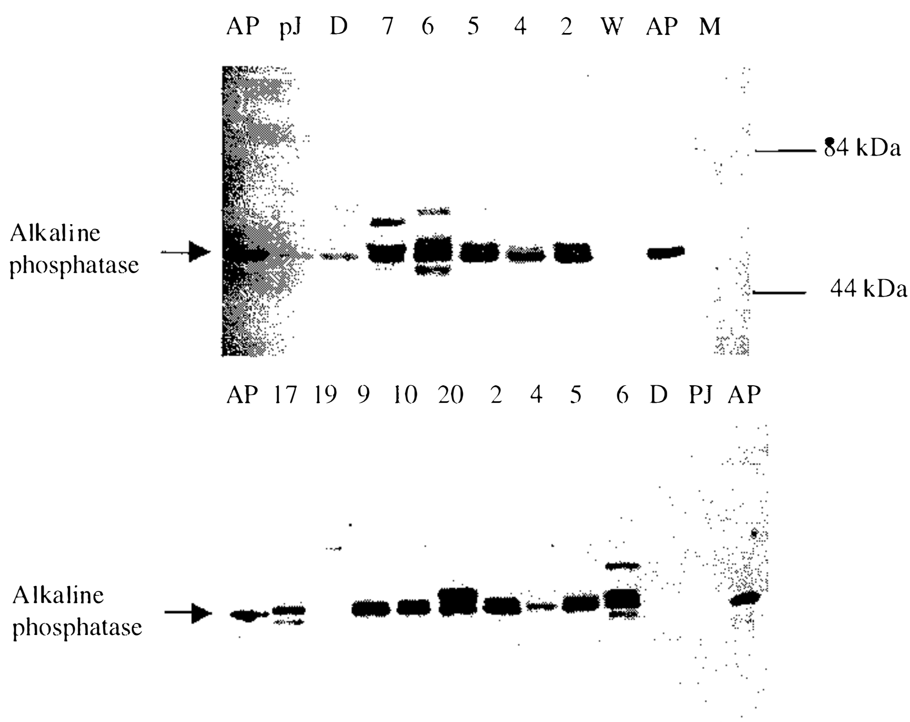


Figure 5.1: Western blot analysis of selected *H. pylori* AP fusion proteins. Periplasmic space (PS) protein was isolated from recombinant *E. coli* and separated by SDS-PAGE. The proteins were transferred to PVDF membrane and screened with a polyclonal antibody raised to alkaline phosphatase and developed using colorimetric detection. PJHp clone # is listed above the corresponding lane; W = pJHp white clone; pJ = vector control (no insert); D = De3 host strain control; AP = alkaline phosphatase control.

same molecular weight as the alkaline phosphatase control or migrating at a higher molecular weight. In some instances, the fusion proteins showed a ladder of degradation products as observed previously (see Chapter 4). These results suggested that the fusion proteins were being degraded by proteolytic digestion either during or after translocation to the periplasmic space. In an attempt to reduce the degradation, the pJEM11 colonies were grown at a lower temperature of 30°C. However, following

analysis by Western blot, the results indicated no change in the levels of degradation (data not shown).

In other experiments, nine periplasmic space protein preparations containing the AP+ fusion proteins were assayed for alkaline phosphatase activity and the levels compared to the activity in the controls which included a non-recombinant *E. coli* clone, a recombinant clone containing pJEM11 with no insert DNA and a recombinant clone containing a pJEM11 *H. pylori* insert that remained white when grown on the AP substrate BCIP (Figure 5.2). Each of the periplasmic fractions from AP+ clones displayed high levels of AP activity ranging from 42.0 units/ml to 286.7 units/ml. Negligible levels of AP activity were detected in each of the controls.

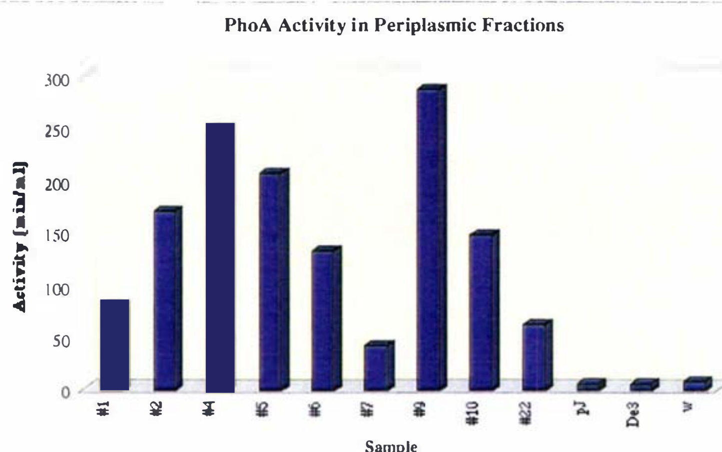


Figure 5.2: Alkaline phosphatase activity in periplasmic space protein fractions isolated from *H. pylori* pJEM11 recombinant clones and controls. # on x axis refer to the pJHp clone number; pJ = pJEM11 control (no insert); De3 = host strain control (no plasmid); W = pJHp white control.

5.4.2 Determination of the purity of the periplasmic space proteins by Western blot analysis

To determine the level of intracellular protein contamination in the periplasmic protein preparations, the intracellular and periplasmic space proteins were isolated from a culture of a number of AP+ transformant clones. Following separation by SDS-PAGE, the proteins from each fraction were stained using Coomassie Brilliant Blue R-250 (BioRad). The protein profiles between the two cell fractions demonstrated distinct differences (Figure 5.3(a)). The fractions were then analysed by Western blot using the alkaline phosphatase antibody as a probe. Strong antibody binding to alkaline

phosphatase was observed in the periplasmic space proteins and in the control AP (Figure 5.3(b)). A small amount of fusion protein was observed in the intracellular

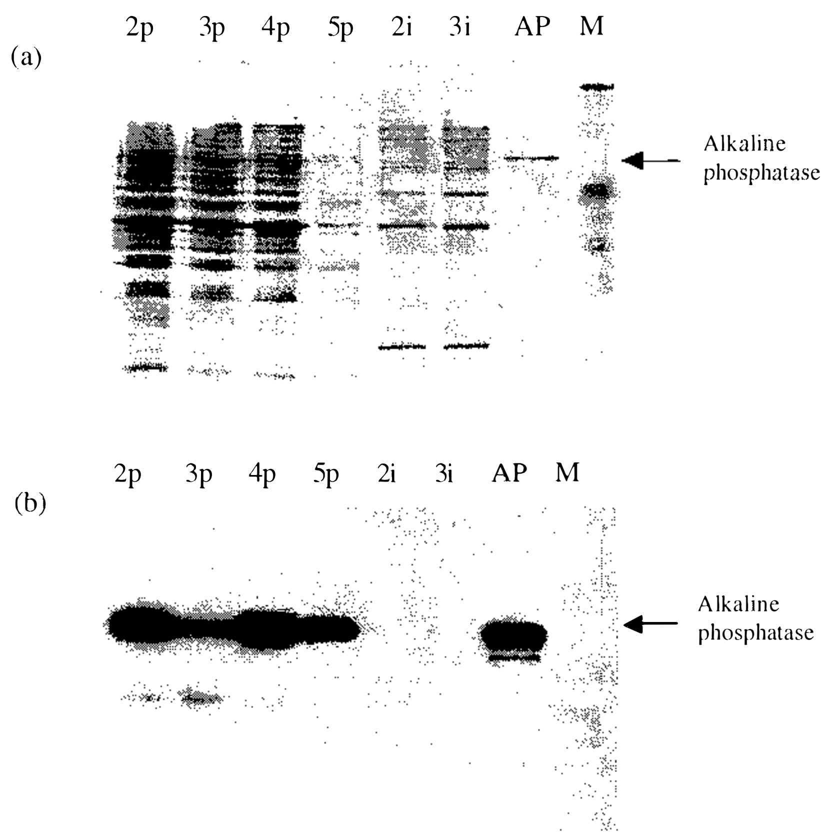


Figure 5.3: (a) Coomassie stain of intracellular and periplasmic space proteins from pJHp blue colonies and alkaline phosphatase control. (b) Autoradiograph of Western blot of intracellular and periplasmic space proteins screened with a polyclonal antibody raised against alkaline phosphatase and developed using chemiluminescence detection. Numbers above the lanes refer to pJHp clone number; AP = alkaline phosphatase; p = periplasmic space protein fraction; i = intracellular protein fraction.

fraction (faint bands), indicating that the major fraction of the fusion protein was recovered in the periplasmic space. The β-galactosidase (β-gal) enzyme, a cytoplasmic protein, was chosen as an intracellular marker to monitor for leakage of cytoplasmic proteins into the periplasmic space. The periplasmic and intracellular proteins from AP+ clones and a pJEM11 vector control were separated on SDS-PAGE and transferred to membrane for Western blot analysis using an antibody raised against β-galactosidase (data not shown). Beta-galactosidase was detected only in the intracellular fraction indicating that there was negligible contamination of the periplasmic space protein fraction with intracellular proteins.

5.4.3 Alkaline phosphatase and β-galactosidase enzyme activity in *E. coli* cellular fractions

The intracellular, periplasmic space and extracellular proteins were then prepared from a number of AP+ clones. The β-gal and AP enzyme activity in each of these fractions was measured. The levels of activity in each fraction was recorded and calculated as a percentage of total activity in each of the fractions (Figure 5.4). In each sample, the

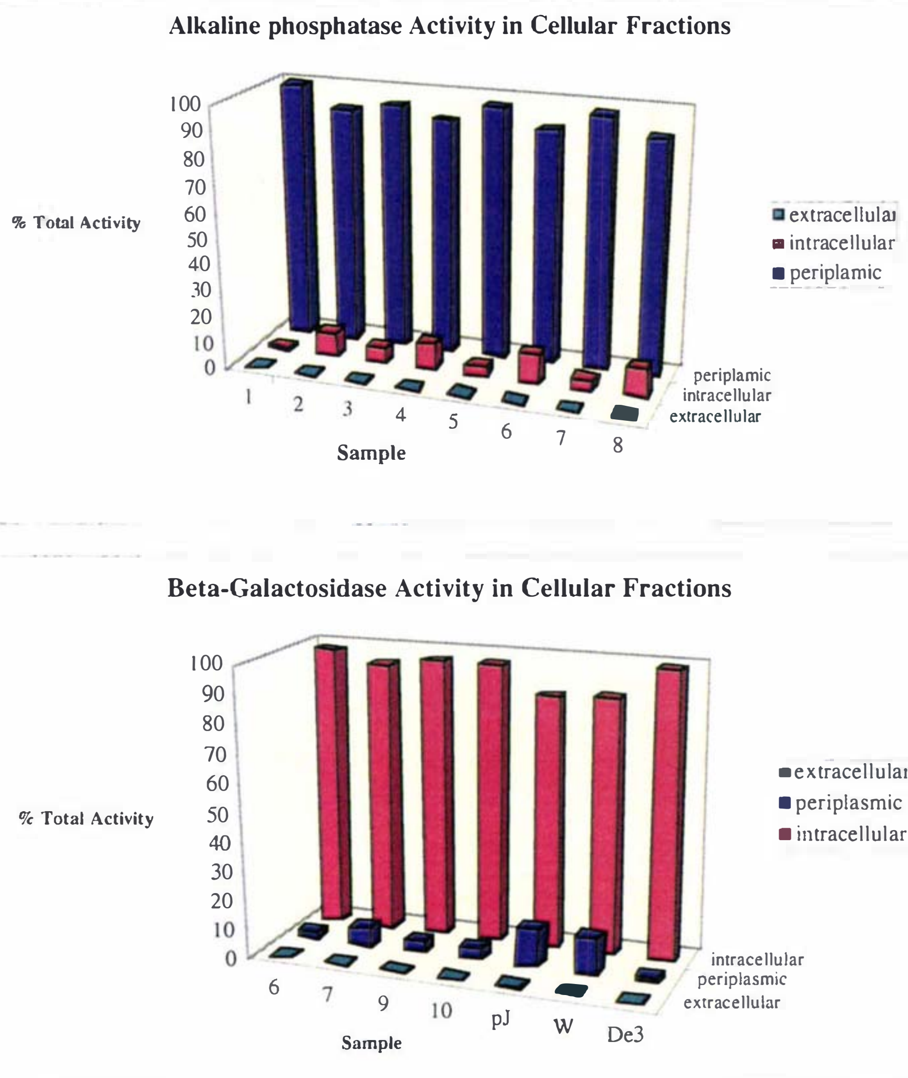


Figure 5.4: Alkaline phosphatase and Beta-galactosidase enzyme assays for cellular fractions derived from *E. coli* pJHp clones and controls. Numbers along the x axis refer to pJHp clone number; pJ = pJEM11 control (no insert), W = pJHp white control; De3 = non-recombinant host strain control.

greatest proportion of the AP activity was detected in the periplasmic space protein fraction and the majority of β-gal activity detected in the intracellular protein fraction.

No enzyme activity from either AP or β-gal could be detected in the extracellular fraction.

5.4.4 Antibody recognition of *H. pylori* AP+ fusion proteins

To investigate the antibody-mediated immune response to some of the exported proteins identified in the pJEMl 1 library, ten periplasmic space fractions enriched for the AP+ fusions were analysed in Western blots using pooled patient sera as the primary antibody from five patients infected with *H. pylori* (Figure 5.5) . A strong band

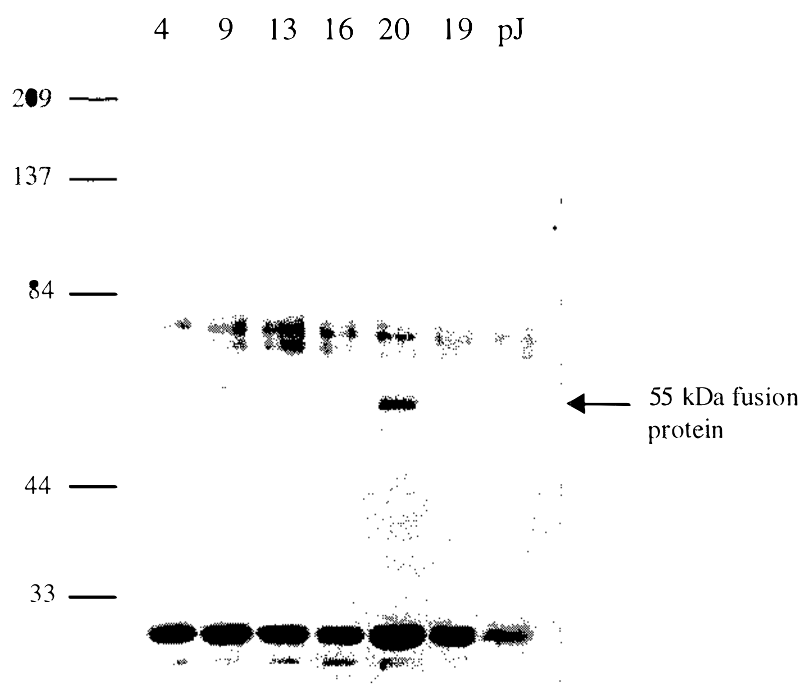


Figure 5.5: Western blot analysis of periplasmic space (PS) protein from *E. coli* pJHp recombinant blue clones. PS protein was separated by SDS-PAGE and transferred to PVDF membrane. The membrane was screened with pooled sera from *H. pylori* infected patients as the primary antibody and developed using colorimetric development. Numbers above the lanes refer to the pJHp clone number; pJ = pJEMl 1 vector control (no insert).

corresponding to an observed molecular weight of 55 kDa was detected in the periplasmic space proteins prepared from *E. coli* pJHp20. A similar band was not observed at this molecular weight in the pJEMl 1 control. Despite the preadsorption of the sera to remove antibodies against *E. coli*, the presence of other common bands on the blot suggested that there was still residual *E. coli* antibodies in the sera. The size of *H. pylori* insert in each of the clones from which periplasmic space proteins was prepared suggested that there was theoretically enough *H. pylori* protein sequence

(ranging from 40 to 286 amino acids) present in the fusion proteins to detect any linear epitopes.

To confirm that the 55 kDa protein corresponded to the AP fusion protein, the periplasmic protein from pJHp20 was electrophoresed in duplicate and transferred to membrane for further Western blot analysis. The blots were screened with an antibody raised against AP and with the pooled sera from infected patients used in the initial screen. An AP fusion protein was detected at the same location as the band reacting with the patient serum, indicating the patient sera contained antibodies to the *H. pylori* protein fused to AP (Figure 5.6). The Western blot protocol was repeated using the

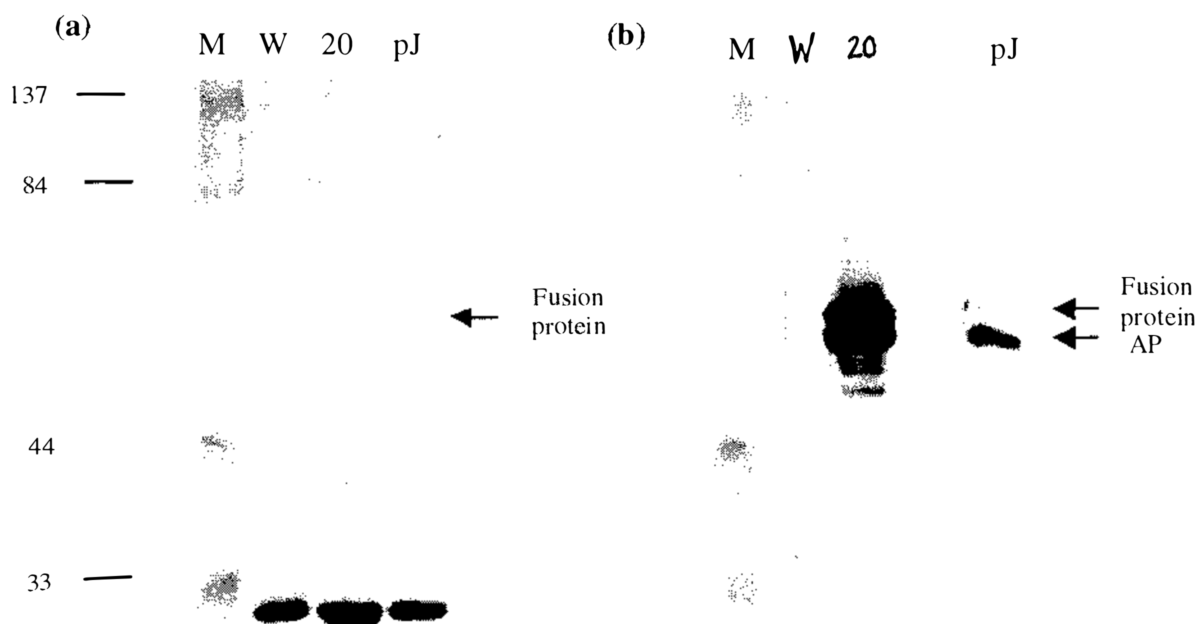


Figure 5.6: Western blot analysis of pJHp20 and controls. (a) Screened with pooled sera from *H. pylori* infected patients (b) Screened with a polyclonal antibody raised against alkaline phosphatase. PS protein and the AP control were separated by SDS-PAGE and transferred to PVDF membrane before screening with the appropriate antibody. Blots were developed using colorimetric detection. M= Kaleidoscope size marker; 20 = pJHp20 clone; W = pJHp white clone; pJ=pJEM1 1 control (no insert).

separated individual sera to determine whether some or all of the patient sera included in the original pool had antibodies that recognised this protein. For each of the individual patient sera, an immunoreactive band at 55 kDa was observed (Figure 5.7). Thus, the epitopes present on this partial *H. pylori* protein appear to be recognised by all of the infected patients from this pool of sera.

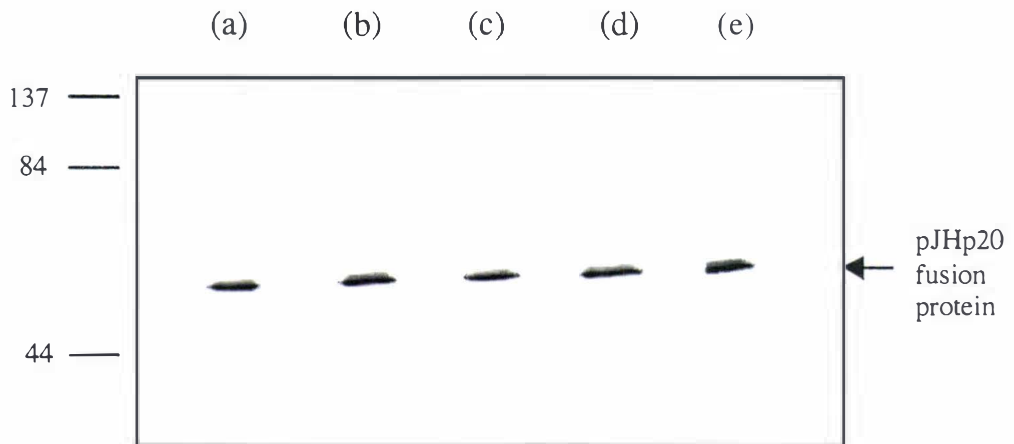


Figure 5.7: Western blot analysis of pJHp20. Periplasmic space protein from pJHp20 was separated on SDS-PAGE and transferred to PVDF membrane. The blot was screened with individual sera from five *H. pylori* infected patients that were used in the original pool. (a) PS025 (b) PS027 (c) PS018 (d) PS014 (e) PS001

5.4.5 Sequence analysis of the plasmid pJHp20

The plasmid encoded by pJHp20 was purified for sequence analysis using primers pJEM6 and pJEM7. The 1.1 kb *H. pylori* insert was found to contain two adjacent ORFs with 98% homology to HP1084 and HP1085 described in the published genome (Tomb *et al.*, 1997) (Figure 5.8). Extensive searches of the current databases failed to find any significant homology with any other sequences available, suggesting that these genes may be unique to *H. pylori*.

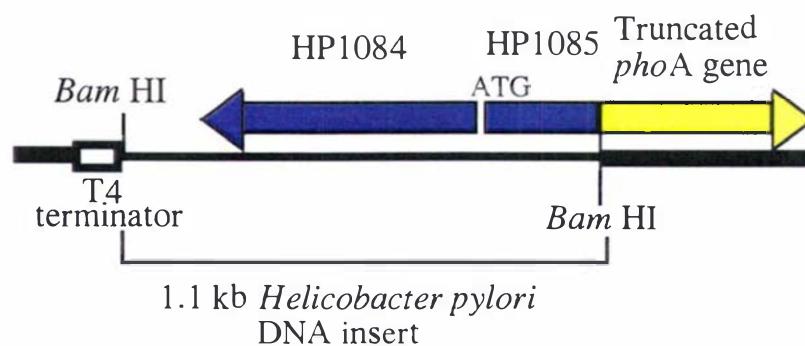


Figure 5.8: Schematic diagram of pJHp20. The plasmid pJEM11 contains a 1.1 kb *H. pylori* insert (blue) fused to the truncated *pho*A gene (yellow). Thick black line represents vector DNA.

5.4.6 Enzyme activity in cellular fractions from cultures of *E. coli* pJHp20

The cellular protein fractions were isolated from *E. coli* containing pJHp20 and the alkaline phosphatase and β-galactosidase activity determined. Cells were harvested at either OD_{600} 1.0 or OD_{600} 1.2 to determine the maximum amount of periplasmic space protein that could be harvested without intracellular contamination. The results are plotted in Figure 5.9 and show that the increase from OD_{600} 1.0 to OD_{600} 1.2 resulted in elevated levels of β-gal activity in the periplasmic space. Thus, for the large scale preparation of further periplasmic space protein from *E. coli*, cells were harvested before reaching an OD of 1.0 (usually in the range 0.7-0.9).

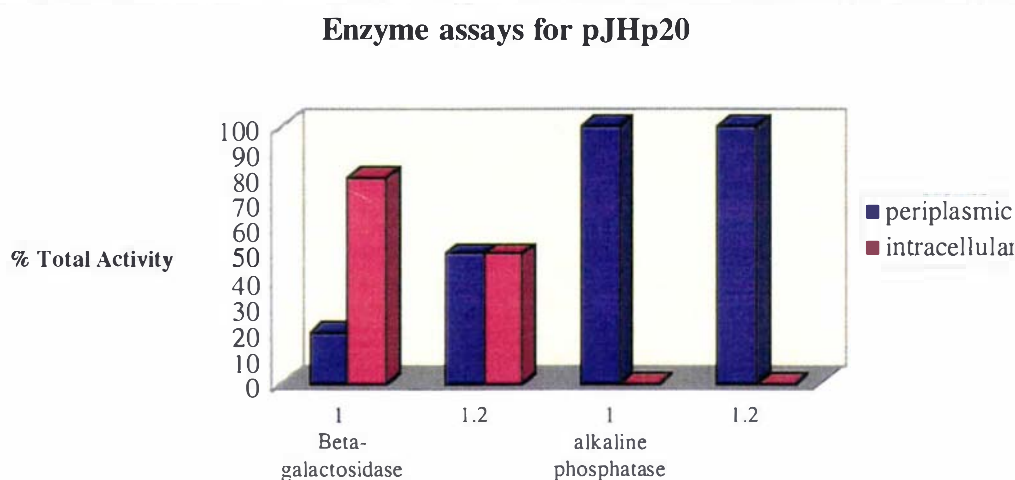


Figure 5.9: Alkaline phosphatase and Beta-galactosidase activity in pJHp20 cellular fractions. Intracellular and periplasmic space proteins were isolated from *E. coli* harbouring plasmid pJHp20 at an culture OD_{600} reading of 1.0 and 1.2. The fractions were then assayed from AP and β-gal enzyme activity and plotted as a precentage of total activity.

5.4.7 Peripheral blood lymphocyte response to *H. pylori* AP+ fusion proteins

To investigate the cellular immune response to individual *H. pylori* proteins, the periplasmic space protein preparations from six *H. pylori* AP+ fusion proteins were incubated with peripheral blood mononuclear cells (PBMC) prepared from four *H. pylori* infected patients. The proliferative responses of PBMCs from infected individuals to the periplasmic space proteins were measured from one patient in the first study (Table 5.1), and from three *H. pylori* infected patients in a separate study (Table 5.2). The availability of the patients dictated the design of this protocol. As positive controls, PBMCs from infected patients were stimulated with the mitogens, SA-1 and

PHA at a predetermined optimal concentration of 1 μg/well. Lymphocytes were cultured along with a broad concentration range of the *H. pylori* fusion preparations (0.5 μg/well to 27.7 μg/well) and cell proliferation measured by colormetric detection. Standard deviations (SD) are shown for the data in Table 5.1, and are representative of the range of SDs observed in each experiment. Each of the periplasmic space protein preparations were concentrated using membrane filtration (Centricon) and standardised for concentration on the basis of measured AP activity. Following incubation with PBMCs, the stimulation index (SI) recorded for the experimental samples showed no significant differences when compared to controls. Therefore, no significant T cell reactivity was detected with any individual patient. In addition, alkaline phosphatase alone did not stimulate proliferation of the lymphocytes. The SI observed with PHA and SA-1 controls indicated that the cells were viable and proliferating.

Table 5.1: Lymphocyte proliferation assays A

Sample (specific activity*)	μg/well	*H. pylori* infected patient		
		Mean OD	**SD**	**SI**
Medium	N/A	536	22	**1.00**
PHA	1.00	1330	20	**2.48**
AP (148.6)	0.78	473	53	**0.882**
	1.56	492	21	**0.918**
	3.13	495	7	**0.924**
	6.25	459	8	**0.856**
	12.5	322	7	**0.601**
PJEM11 (0.2)	0.78	483	3	**0.902**
	1.56	477	17	**0.890**
	3.13	490	5	**0.915**
	6.25	467	10	**0.871**
	12.5	478	-	**0.892**
PJHp4 (4.4)	0.78	439	31	**0.820**
	1.56	493	8	**0.919**
	3.13	477	-	**0.890**
	6.25	439	2	**0.819**
	12.5	459	12	**0.857**
PJHp9 (6.0)	0.78	477	6	**0.890**
	1.56	494	32	**0.921**
	3.13	518	19	**0.967**
	6.25	489	-	**0.913**
	12.5	469	15	**0.876**
PJHp20 (1.0)	0.78	491	11	**0.916**
	1.56	510	31	**0.952**
	3.13	516	6	**0.963**
	6.25	510	4	**0.952**
	12.5	501	-	**0.934**

N/A: Not applicable; **Mean OD**: average OD_{570} reading from triplicate wells x 1000; **SD**: observed standard deviation; **SI**: stimulation index; *Specific Activity (units/min/mg/ml)

Table 5.2: Lymphocyte proliferation assays B

Sample (specific activity*)	µg/well	*H. pylori* infected 1		*H. pylori* infected 2		*H. pylori* infected 3	
		Mean OD	SI	Mean OD	SI	Mean OD	SI
Medium	N/A	632	**1.00**	570	**1.00**	287	**1.00**
PHA	1.00	1163	**1.84**	947	**1.66**	991	**3.46**
SA-1	0.88	1414	**2.24**	1801	**3.16**	928 (1 µg/well)	**3.23**
	0.44	984	**1.56**	808	**1.42**		
	0.22	771	**1.22**	500	**0.88**		
	0.11	1064	**1.68**	209	**0.37**		
AP (148.6)	0.83	16	**0.03**	22	**0.04**	45 (0.1 unit/well)	**0.16**
	0.42	28	**0.04**	126	**0.22**		
	0.21	362	**0.57**	446	**0.78**		
	0.10	592	**0.94**	486	**0.85**		
pJEM11 (0.3)	22.90	478	**0.76**	495	**0.87**	ND	ND
	11.45	514	**0.81**	526	**0.92**		
	5.73	539	**0.85**	566	**0.99**		
	2.86	556	**0.92**	567	**0.99**		
PJEM11 white (0.4)	15.70	503	**0.80**	514	**0.90**	252	**0.88**
	7.85	511	**0.81**	546	**0.96**	313	**1.09**
	3.93	534	**0.84**	569	**1.00**	415	**1.45**
	1.96	552	**0.87**	573	**1.00**	440	**1.53**
	0.98	ND	ND	ND	ND	439	**1.53**
	0.49	ND	ND	ND	ND	376	**1.31**
PJHp6 (7.9)	14.80	441	**0.70**	432	**0.76**	ND	ND
	7.40	466	**0.74**	502	**0.88**		
	3.70	511	**0.81**	545	**0.96**		
	1.85	548	**0.87**	556	**0.98**		
PJHp7 (1.9)	27.70	509	**0.81**	519	**0.91**	236	**0.82**
	13.85	520	**0.82**	550	**0.96**	324	**1.13**
	6.93	543	**0.86**	562	**0.99**	388	**1.35**
	3.46	556	**0.88**	549	**0.96**	397	**1.38**
	1.73	ND	ND	ND	ND	410	**1.43**
	0.865	ND	ND	ND	ND	381	**1.33**
PJHp9 (5.8)	8.00	501	**0.79**	548	**0.96**	ND	ND
	4.00	538	**0.85**	590	**1.03**		
	2.00	592	**0.94**	593	**1.04**		
	1.00	663	**1.05**	587	**1.03**		
PJHp10 (3.8)	15.00	490	**0.78**	516	**0.90**	250	**0.87**
	7.50	513	**0.81**	571	**1.00**	338	**1.18**
	3.75	546	**0.86**	589	**1.03**	386	**1.35**
	1.88	589	**0.93**	588	**1.03**	414	**1.44**
	0.94	ND	ND	ND	ND	375	**1.31**
PJHp4 (3.5)	17.4	ND	ND	ND	ND	257	**0.90**
	8.7					305	**1.06**
	4.35					400	**1.40**
	2.18					429	**1.50**
	1.09					391	**1.36**
	0.54					378	**1.32**

N/A: not applicable; **ND**: not done; **mean OD**: average OD_{570} reading from triplicate wells x 1000; **SI**; stimulation index; *Specific Activity (units/min/mg/ml)

5.5 Discussion

The study reported here describes a preliminary immunological characterisation of exported proteins of *H. pylori*, identified and partially purified as alkaline phosphatase gene fusions. The results obtained from the enzyme activity analyses in conjunction with Western blotting, demonstrated that signal peptides of *H. pylori* are functional and recognised by the export machinery of *E. coli*, and that the AP fusion proteins were transported to the periplasmic space in *E. coli*. The periplasmic space protein, enriched for the *H. pylori* AP+ fusion proteins were therefore screened with patient antibody in Western blot assays and tested against peripheral blood mononuclear cells for T cell recognition. The individual AP+ fusion proteins in the periplasmic space protein preparations were initially identified in Western blot assays using an antibody raised against AP. These size of the bands on these blots confirmed that there was sufficient *H. pylori* amino acid sequence present in each AP+ fusion protein to detect potential protein epitopes that may be present.

Sequence analysis, in conjunction with the release of the completed *H. pylori* genome sequence, facilitated characterisation of the cloned inserts. Each of the AP+ fusion proteins tested contained *H. pylori* amino acid (aa) sequence fused to AP, ranging from 40 aa to 286 aa. Plasmid pJHp20 was chosen for further investigation because it contained inserts expressing polypeptide epitopes recognised by patient antibody. Amongst the other AP+ fusion proteins screened, two (pJHp5/9 and pJHp10) contained a region of coding sequence lacking the amino terminus and start codon of the respective genes. Therefore, the size of the *H. pylori* protein fused to AP (216 aa and 94 aa respectively) was deduced from the predicted internal start site (see Chapter 4 of pJHp5/9). In addition, two of the *H. pylori* inserts contained the start regions of HP0823 (pJHp6) and HP0243 (pJHp13) fused to an intervening Sau3A fragment from another gene. Three other clones, pJHp3/4, pJHp7 and pJHp16 contained 70, 55 and 286 predicted amino acids respectively, fused to AP. However, because of suspected proteolytic degradation, the amount of predicted peptide sequence may not have been equivalent to that tested in the immunoassays.

Although alkaline phosphatase gene fusions have been successfully screened for immunoreactive T cell epitopes (Moran *et al.*, 1999; Borich, 1997), there are several

potential disadvantages to using fusion proteins for screening with antibodies. One possibility is that the structural and conformational integrity of the fused polypeptide may differ considerably from the same polypeptide in the native protein. This could have consequences for presentation of epitopes recognised by antibody. This may explain why only one *H. pylori* AP fusion protein was found to react with patient antibody. The majority of antibodies recognise epitopes that depend upon the conformation of the native protein (Blundell *et al.*, 1987). This may vary depending upon the nature of the antigen, as some cell surface proteins for example are likely to be formed by discontinuous surface sequences (Horsfal *et al.*, 1991). The detection of B cell epitopes using SDS-PAGE is therefore limited to the identification of linear epitopes that are not dependent on native conformation. An alternative explanation for the lack of recognition of the fusion porteins is that the proportion of antibodies in the serum, specific for limited number of epitopes, may have been outside the detection limit of the Western blot assay.

Recogntion of antigen by T helper cells requires processing by antigen presenting cells and subsequent presentation of short linear peptides to the appropriate T cell. There is evidence that peptides of less than 12 amino acids can bind to T-cell receptors when presented in the context of the appropriate major histocompatablity complex (MHC) (Horsfall *et al.*, 1991). Thus, it is conceivable that even short *H. pylori* sequences attached to a reporter protein could be recognised by lymphocytes previously exposed to the antigen. Considering the smallest amount of *H. pylori* protein fused to AP was 20 amino acids, each of the fusion proteins tested had the potential to contain T cell epitopes. In spite of this, none of the six *H. pylori* AP+ fusions stimulated proliferation of PBMCs from four *H. pylori* infected patients. PBMC'S from a larger population of *H. pylori* infected and non-infected patients would need to be tested to reach any conclusions. It is possible, however, that the *H. pylori* polypeptides, expressed as fusion proteins, were not immunodominant and recognised by the PBMC from the infected patients. Alternatively, they could be antigenic but the PBMC from the infected patients contained only a very small proportion of antigen specific T cells primed to recognise the particular polypeptide epitopes. The proliferation of these cells may consequently have been outside the detection limit of the assay.

The suppression of T cell responses by *H. pylori* antigens has been observed previous studies (Knipp *et al.*, 1993; Birkholz *et al.*, 1993). The design of the assays did not allow us to specifically address the phenomenon of suppressed T cell responses in these patients, however, it is a possibility that cannot be ruled out. A study has shown that a recombinant fusion protein carrying an immunodominant region of CagA affected the proliferation of human cells. This fusion protein not only inhibited PHA-driven T cell proliferation, but also inhibited growth of macrophages and epithelial cells from metaplastic gastric carcinoma. CagA therefore has anti-proliferative activity that is not restricted to T cells (Rudnicka *et al.*, 1998). The present study also used a population of lymphocytes isolated from peripheral blood. It could be that gastric lymphocytes, at the focus of infection, would constitute a more appropriate population for proliferation assays. It may be that T cells that respond to *H. pylori* in infected persons are compartmentalised in the GI tract, while peripheral T cells reflect prior systemic exposure. However, it has been demonstrated that there is no difference between peripheral blood lymphocytes and gastric lymphocytes when comparing proliferative responses to *H. pylori* between *H. pylori* seropositive and seronegative patient derived T cells (Fan *et al.*, 1994). Varying distribution of HLA class II phenotypes in people from different populations can also influence peptide reactivity (Roche *et al.*, 1996) and may account for the lack of a T cell response to these antigens in the four subjects from which PBMC were taken.

Although the putative outer membrane protein identified in this study appears to contain B cell epitopes, it did not induce proliferation of T cells from *H. pylori* infected patients. Dichotomy in the B cell and T cell responsiveness to the same antigen in *H. pylori* infected patients has been observed (Sharma *et al.*, 1994). However, since only a small proportion of the entire protein was fused to AP, there is a possibility that other immunodominant epitopes are present in other regions of the protein capable of stimulating B and T cells. In evaluating the usefulness of immunoscreening recombinant fusion proteins from *E. coli* with patient antibody, it appears that the methodology described in this study has proved a rapid way of detecting antigens that might be involved in the immune response against *H. pylori*, or could be useful as immuno-diagnostic reagants.

Chapter 6 Cloning and Heterologous Expression of an Immunogenic Protein from *H. pylori*

6.1 Abstract

The application of alkaline phosphatase (AP) fusion methodology was used to identify *Helicobacter pylori* exported proteins. Serological screening of these AP+ fusion proteins identified a gene with a partial open reading frame (ORF) encoding a highly immunogenic, putative exported protein. The predicted amino acid sequence of this gene displays a characteristic N-terminal signal peptide and also contains regions of C-terminal hydrophobicity consistent with a membrane-associated protein. Southern blot analysis revealed that the gene was absent in other *Helicobacter* species tested. In addition, by use of a combination of PCR and sequence analysis of the amplified gene product, it was shown to be highly conserved amongst *H. pylori* isolates. In order to obtain pure recombinant protein, the gene encoding the protein was cloned and expressed in *Escherichia coli* as a Beta-galactosidase (β-gal) fusion and the protein was then purified by affinity chromatography and proteolytic cleavage of the β-gal moiety. In Western blot assays, the purified protein (with an apparent molecular weight of 18 kDa) was recognized by 71% of sera taken from patients infected with *H. pylori*, but by only 16% of sera taken from patients with unrelated or no gastrointestinal disease. These results indicated that the 18 kDa protein from *H. pylori* was expressed *in vivo* and was immunogenic. The purified antigen was also tested in a murine model of *H. pylori* infection to determine its ability to generate a protective immune response. Oral administration of this antigen with a cholera toxin adjuvant did not protect mice against infection following challenge with *H. pylori*.

6.2 Introduction

Helicobacter pylori infection in humans is responsible for a number of clinically significant diseases including peptic ulcer disease and gastric adenocarcinoma. Although there are effective treatments currently available to cure *H. pylori* infection (Axon, 1998), the lack of a simple, cheap antibiotic regime and the emergence of antibiotic resistant strains (Megraud, 1998) makes the development of a vaccine a desirable goal.

The data obtained from previous vaccination studies using animal models of *Helicobacter* infection have provided evidence that many of the protective antigens are found among the secreted or surface proteins of *H. pylori* (reviewed in Vyas and Sihorkar, 1999). Oral immunisation of mice with *H. pylori* bacterial sonicates, or recombinant antigens, along with cholera toxin as an adjuvant, results in the induction of specific IgG-secreting cells that correlate with protection against *H. felis* infection (Ferrero *et al.*, 1995; Ferrero *et al.*, 1997). Some examples of surface-exposed *H. pylori* proteins considered to be protective antigens include the urease enzyme (Michetti *et al.*, 1994; Ferrero *et al.*, 1994; Pappo *et al.*, 1995), heat shock proteins (Ferrero *et al.*, 1995), and the vacuolating cytotoxin, VacA (Marchetti *et al.*, 1995). A recent study (Hocking *et al.*, 1999) has identified other potential vaccine candidates, including the lipoprotein Lpp20 (Kostrzynska *et al.*, 1994). This lipoprotein has been identified as a component of outer membrane vesicles (OMVs) released from *H. pylori* under certain growth conditions. In mouse vaccination trials, OMVs containing Lpp20 induce protection against infectious *H. pylori* challenge (Keenan *et al.* submitted for publication).

The *H. felis* mouse model has been widely used to evaluate vaccine candidates. *H. felis* was originally isolated from the stomach of a cat, and in some strains of mice, causes disease pathology similar to that seen in humans with *H. pylori* infection (Lee *et al.*, 1990, 1993). One advantage of using this model to screen candidate *H. pylori* vaccine antigens is that many antigens are conserved between the two *Helicobacter* species. However, a limitation of this model is that *H. felis* does not produce VacA and CagA, two important *H. pylori* virulence factors. Therefore, the validity of using *H. felis* as a surrogate for *H. pylori* is open to question.

A more relevant mouse model has been described for testing the safety and efficacy of potential vaccine candidates against challenge with *H. pylori* (Lee *et al.*, 1997). This mouse adapted *H. pylori* strain, known as the Sydney strain (SSI), colonises the mouse to high levels of infection, exhibits specific adhesion to gastric epithelial cells and also causes pathology resembling that seen in humans infected with *H. pylori.* In addition, the use of the standardised *H. pylori* "Sydney Strain" (Lee *et al.*, 1997) in challenge experiments allows for direct comparisons with other immunisation protocols.

In Chapter 4 the use of alkaline phosphatase gene fusions to identify *H. pylori* genes encoding secreted or membrane-associated proteins was described. The follow-up study (chapter 5) described the immunological screening of these partial surface and secreted *H. pylori* proteins fused to AP, and led to the identified of a previously uncharacterized *H. pylori* exported protein that was recognised by sera from *H. pylori* infected patients. The present study describes further investigations into this immunogenic protein, to determine its potential as a candidate for inclusion in a subunit vaccine against *H. pylori* infection. The gene encoding the protein (HP1085) was investigated for its distribution among *H. pylori* clinical isolates and other *Helicobacter* species. The entire protein, with a predicted molecular weight of 18 kDa, was expressed and purified from *E. coli*. The recombinant protein was then tested for its immunogenicity against a panel of sera taken from patients positive or negative for *H. pylori* infection and for its protective efficacy in a *H. pylori* mouse model of infection.

6.3 Materials and Methods

6.3.1 Polymerase chain reaction

Genomic DNA prepared from eight *H. pylori* clinical isolates, *H. pylori* CCUG 17874, *H. mustelae* 4298 and *H. felis* (see Chapter 2.3.3) was used as the template in the polymerase chain reaction. Oligoncucleotide primers were designed to amplify the ORF (HP1085) encoding the 18 kDa protein, based on the full sequence from the *H. pylori* 26695 genome (Tomb *et al.*, 1997). The primers were designed to produce a truncated HP1085 sequence beginning 5 residues from the putative cleavage point of the signal sequence with an *Eco* RI site incorporated into the 5' end and a *Kpn* I site at the 3' end. The sequence of the primers were as follows:

Primer 20/1: 5' TAATGAATTCACTTACACGCTAGAC 3'

Primer 20/2: 5' TTGAGGTACCGGGACTTGATAAGC 3'

High fidelity Pwo DNA polymerase was used in the PCR with approximately 20 ng of genomic DNA as template. PCR amplification consisted of an initial denaturation step (95°C, 5 mins) followed by 30 cycles of amplification consisting of denaturation (95°C, 30 sec), annealing (50°C, 30 sec) and primer extension (72°C, 1 min). The PCR products generated were separated on a 1% agarose gel and purified using a Qiagen gel purification kit (QIAGEN) for sequencing. The purified PCR products (approximately

400 ng) were sequenced by automated sequencing using primer 20/1 at a concentration of 8 pmol.

6.3.2 Southern blot analysis

For Southern blotting, approximately 5 µg of genomic DNA from *Helicobacter* sp. was digested with *Hin*d III and separated by electrophoresis on a 1% agarose gel and the DNA transferred to positively-charged membrane (Amersham, UK) by capillary action (Chapter 2.8.2). The PCR amplification product generated from *H. pylori* CCUG 17874 was purified from an agarose gel followed by phenol/chloroform extraction for use as a probe in Southern blots. The probe was labeled with DIG and quantitated according to the manufacturer's instructions (Chapter 2.8.1). Following prehybridization at 42°C for four hours, the blot was hybridized with 25 ng probe/ml of hybridization solution (DIG Easy Hyb, Boehringer Mannheim) overnight at 42°C. The blot was washed under moderate stringency using 1x SSC at 60°C and developed with anti-DIG antibody conjugated to alkaline phosphatase. After 30 mins incubation in substrate, the blot was developed by autoradiography.

6.3.3 Expression and purification of the 18 kDa protein

6.3.3.1 Cloning of the HP1085 ORF into expression vectors

The pXa series of plasmids (Boehringer Mannheim) were used for initial expression of the HP1085 ORF. These plasmids contain the *lac* promoter and a multiple cloning site in all translational frames of the *lacZ* gene to create β-galactosidase fusion proteins. A PCR product was generated from CCUG 17874 using primers 20/1 and 20/2 (section 6.3.1) and purified from an agarose gel. The PCR product and expression vector were digested with the restriction enzymes *Eco* RI and *Kpn* I and purified from a 1% agarose gel. The digested PCR product and plasmid were ligated using 1 unit of T4 ligase for 1 hour at 22°C and transformed into the *E.coli* strain XLI-blue by heat shock at 42°C for 90 seconds. Recombinant colonies were grown on selective media containing 100 µg / ml of ampicillin from which eight were selected and resuspended in 100 µl of water. Total DNA was prepared by boiling the suspension for 10 minutes, followed by centrifugation to remove cell debri. Five microlitres of the supernatant was removed and used in the PCR with primers 20/1 and 20/2 to confirm the presence of the insert.

Beta-galactosidase derived polypeptides can be isolated on the basis of their affinity for the lactose analogue APTG. In practice, this method yields material of excellent purity, but is not very efficient and is ineffective for purification of proteins of low abundance. To obtain large amount of recombinant protein for the mouse immunisation trials, the 18 kDa protein was also prepared as a His-tagged recombinant protein. The *Eco* RI and *Kpn* I fragment was cloned into the compatible sites of the expression vector pPROEX Htb (Life Technologies) as described above.

6.3.3.2 Expression and purification of the 18 kDa protein

Positive colonies were selected and grown in LB broth containing 100 μg / ml ampicillin at 37°C. Expression of the fusion protein was induced by the addition of isopropyl-β-δ-thiogalactopyranoside (IPTG) into the culture medium when an OD_{600} 0.8 was reached. Varying amounts of IPTG (0.5 mM – 2.0 mM) was added to the culture to determine the optimal levels of induction, over periods ranging from 1 hour to overnight. The cells were then harvested by centrifugation at 6,000 rpm and resuspended in loading/washing buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 1.6 M NaCl, 10mM 2ME, pH 7.4). The fusion protein was released by sonication, and the supernatant applied to an APTG (4-aminophenyl-β-δ-thiogalactopyranoside) affinity chromatography column according to the manufacturer's instructions (Boehringer Mannheim). The fusion protein was eluted with 0.1 M borate, 10 mM 2ME, pH 10 and the fractions containing the highest amount of protein, as determined by SDS-PAGE, were pooled. The β-gal portion of the fusion was removed by proteolytic cleavage with Factor Xa (biotin-labeled) at different concentration ratios of enzyme:substrate (1:10, 1:150 and 1:100) over time at 25°C. The enzyme was subsequently removed by incubating with streptavidin gel for 30 mins at RT. The gel was removed by centrifugation at 14,000 rpm and the cleaved β-gal portion removed by a second APTG affinity chromatography step yielding pure recombinant protein.

For purification of the his-tagged recombinant protein, 0.2 mM IPTG was added to the culture medium and the cells harvested after 6 hours induction and resuspended in start buffer (20 mM Na_2HPO_4, 0.5 M NaCl, pH 7.4) with the addition of 20 mM imidazole. The resuspended cells were sonicated, cell debri removed by centrifugation at 14,000 rpm and the soluble fraction removed. Purification of the recombinant protein was

achieved using a nickel chelating affinity column as described in Chapter 3.3.6. Following the application of the sample, contaminants binding to the column were eluted by washing with Start buffer containing 40 mM imidazole, followed by a 60 mM imidazole wash. The recombinant protein was eluted using 200 mM imidazole and the purity of the protein determined by SDS-PAGE. Protein concentration was estimated by reading the absorbance at 280 nm taking into account the extinction coefficient of the truncated HP 1085 amino acid sequence along with the histidine hexamer tag.

6.3.3.3 N-terminal sequencing of the recombinant 18 kDa protein

Twenty microlitres of the pure recombinant protein cleaved from β-gal was electrophoresed on a 12.5% SDS-PAGE gel and blotted onto PVDF membrane as previously described (Chapter 2.10.2). The membrane was subsequently stained with 0.25% Coomassie brilliant blue and excised for N-terminal sequencing. The N-terminal amino acid sequence analysis was performed with a pulse liquid phase sequenator (Model 477A, Applied Biosystems) by the Amino Acid Analysis and Protein Sequencing Service, Massey University, NZ.

6.3.4 Western blot analysis

Electrophoresis of proteins was routinely performed on SDS-polyacrylamide gels using standard methods using BioRad Mini Protean II apparatus according to the manufacturer's instructions and as previously described (Chapter 2.10.2). For Western blotting, approximately 20 μg of purified protein/lane was run on a 12% SDS-PAGE gel and transferred to PVDF membrane using a TransBlot semi-dry transfer cell (BIORAD) according to the manufacturer's instructions. After blocking, the blots were incubated in patient sera diluted 1:200 in PBS for two hours at RT using Biorad mini-protean II multi-screen apparatus. Following washing, the membranes were incubated at RT for 2 hours in anti-human IgG alkaline phosphatase conjugated secondary antibody (Sigma, St. Louis) diluted 1:20,000 in PBS. The color reaction was performed by developing with 0.05 mg/ml of BCIP and 0.01% nitroblue tetrazolium (NBT). Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford).

6.3.5 Patient samples

Sera was collected from thirty-two individuals at the Wakefield Gastroenterology Centre, Wellington (summarised in Table 2.4). Of the total thirty-two individuals from whom samples were collected, twenty-one were determined to be infected with *H. pylori*. Infection was diagnosed by either CLOtest, urease breath test (UBT) and/or by testing for anti-*H. pylori* antibodies in serum (Quikview Kit). Control sera were obtained from eleven asymptomatic volunteers who were determined to be *H. pylori* negative by Quikview and CLOtest (three) or by Quikview only (eight). Serum samples were stored at –70°C until immunoblotting was performed.

6.3.6 Mouse immunisation protocol

C57B1/6J mice were purchased from the Animal Breeding Station, University of Otago, and housed at the Wakefield Gastroenterology Research Centre, Wellington. All mice were maintained under conventional conditions in an isolation unit and were 8-10 weeks of age at the time of the experiment. Experimental mice (n= 6) were immunised orogastrically four times at fortnightly intervals with recombinant protein plus cholera toxin (10μg)(Sigma, St. Louis). Each mouse was initially immunised with 750 μg of recombinant 18 kDa antigen, followed by 500 μg, 500μg and 250 μg doses for the next three immunisations respectively. As controls, mice (n=6) were immunised with 1 mg/ mouse/immunisation of *H. pylori* sonicate extract (HPSE) plus CT (10 μg) or PBS plus CT (10 μg). The mouse-adapted *H. pylori* Sydney strain 1 (SS1), established by Lee *et al.*, (1997) was used for challenge. Mice were inoculated orally with 1 x 10^8 colony-forming units (CFU) of freshly harvested *H. pylori* in 500 μl BHI broth by using a 19-gauge feeding needle as described previously (Chen *et al.*, 1993). Mice were sacrificed by overdose of carbon dioxide at 6 weeks post-challenge, and the serum and stomachs of the animals were collected for analysis.

The presence of *H. pylori* infection in the individual mice was determined by biopsy urease test and quantitative bacterial culture. After removing the contents, the stomach was washed in sterile physiological saline and placed on a flat filter paper with mucosal side up. They were then dissected longitudinally along the greater curvature into three tissue fragments so that each fragment contained gastric cardia, body and antrum. For

each stomach, one fragment was used for determination of the presence of urease activity. The tissue was placed in urea-containing medium and incubated at RT for 5 hours. A red-pink colour was recorded as positive and the sensitivity of the test was approximately $\log_{10}$ 5.0 CFU/g stomach tissue. The second set of tissue fragments was used for quantitative bacterial cultures. The tissue was homogenised in brain heart infusion (BHI) broth, serially diluted and plated onto agar plates containing Glaxo Selective Supplement A (Lee *et al.*, 1997). The plates were incubated for 72-96 hours under microaerophillic conditions and the number of colonies counted and expressed as CFU per gram of tissue. With this method, $\log_{10}$ 2.6 CFU per gram of stomach represented the limit of detection.

Antibodies specific for *H. pylori* were measured by an enzyme-linked immunosorbent assay modified from a previously described procedure (Chen *et al.*, 1993). Briefly, 96-well Immunolon 4 plates (Dynex Technologies, VA) were coated with 20 µg/ml; 50 µl/well, of recombinant HP1085-His protein or whole cell extracts of *H. pylori* SSI in carbonate-bicarbonate buffer (pH 9.5) at 4°C overnight. The plates were blocked by incubation with 1% Gelatin in PBS at RT for 1 hour and then rinsed four times with PBS with 0.05% Tween 20. 100 µl of pre-diluted serum samples (1:50) were added to the wells and the plates were incubated at RT for 3 hours. An alkaline phosphatase conjugated secondary antibody specific for mouse IgA and IgG (Sigma, St. Louis) was added for 2 hours at RT. Colour reactions were developed by addition of p-nitrophenyl phosphate (pNPP) or 2,2'-azinobis (3-ethylbenxthiazolinesulfonic acid) substrates (Sigma) and the optical density measured at 405 nm with an automated ELISA plate reader.

6.3.7 Statistical Analysis

Data are presented as means $\pm$ standard deviations (SD) for parametric data and median ranges for non-parametric data. Differences in the bacterial colony counts between groups of animals and proliferation assay results were determined by Student t test. Differences in antibody titres were determined by the Mann-Whitney U test. Differences were considered significant for a P value of ≤ 0.05.

6.4 Results

6.4.1 Distribution of the HP1085 gene among *Helicobacter* species

A panel of independent *H. pylori* isolates representing Type I and II *H. pylori* strains were tested for the presence of the HP1085 gene encoding the 18 kDa protein by PCR amplification. The HP1085 gene was detected as a 444 bp product in all ten *H. pylori* isolates tested (see Table 2.1 for details on *H. pylori* strains). Neither *Helicobacter felis* nor *Helicobacter mustelae*, the *Helicobacter* species naturally colonising the stomach of the cat and ferret respectively, produced a PCR product (Figure 6.1). Southern blot experiments were also carried out to test for the presence of the HP1085 gene in chromosomal DNA from other *Helicobacter* species using the 444 bp PCR

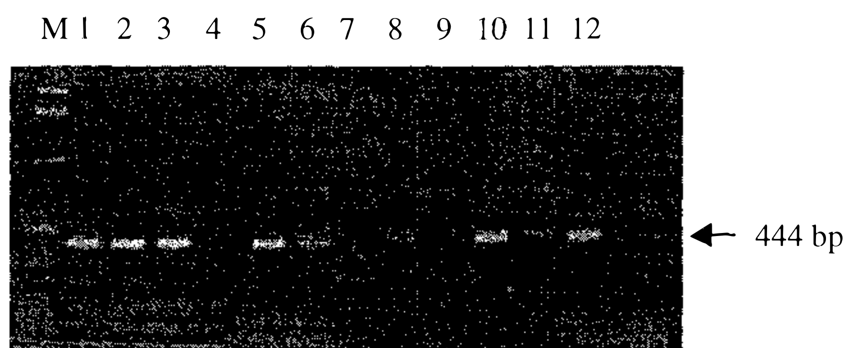


Figure 6.1: PCR amplification of the HP1085 gene from *Helicobacter* species. Primers 20/1 and 20/2 were used in the PCR with genomic DNA prepared from *H. pylori* strains, *H. mustelae* and *H. felis*. Lane1, *H. pylori* MU005; lane 2, *H. pylori* MU007; lane 3, *H. pylori* MU008; lane 4, *H. felis*; lane 5, *H. pylori* MU015, lane 6, *H. pylori* MU016, lane 7, *H. mustelae*; lane 8, *H. pylori* MU038; lane 9, no DNA control; lane 10, *H. pylori* MU064; lane 11, *H. pylori* MU091; lane 12, CCUG 17874. Products were separated on a 1% ethidium bromide agarose gel and visualised under UV light. M, 1kb marker.

amplification product amplified from *H. pylori* CCUG 17874 as a probe. The results of the PCR were consistent with the absence of the HP1085 gene sequence in the chromosome of these species. No hybridization was observed with chromosomal DNA from *H. felis* or *H. mustelae*, however, a strong signal was detected with *H. pylori* 17874 (Figure 6.2). The results of this screen suggest that the HP1085 gene is present in *H. pylori*, but absent in other *Helicobacter* species. Searches of DNA and protein

databases to identify similarities with HP1085 revealed no homologies in the current databases, suggesting that this gene may be unique to *H. pylori*.

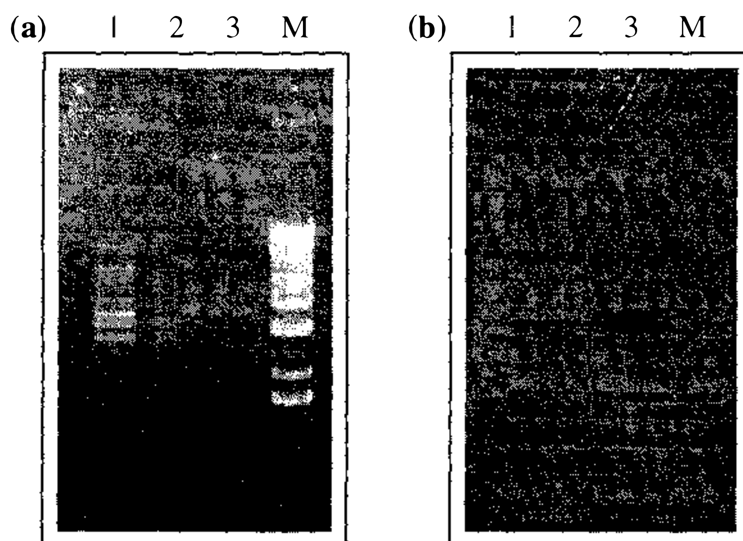


Figure 6.2: Southern blot of genomic DNA from *Helicobacter* species, using the HP1085 gene PCR product as a probe. (a) Agarose gel. Genomic DNA was isolated from *Helicobacter* sp., digested with the restriction enzyme *Hin*d III and separated on a 1% agarose gel. (b) Autoradiograph of Southern blot. Transferred DNA was probed with radiolabelled HP1085 as described in section 6.3.2. Lane 1, *H. mustelae*; lane 2, *H. felis*; lane 3, *H. pylori* 17874; M, Lambda DNA marker (Life Technologies).

6.4.2 Conservation of the HP1085 gene among *H. pylori* strains

The PCR product from five *H. pylori* clinical isolates and the *H. pylori* CCUG type strain 17874 was sequenced. The resulting sequences from these five clinical strains, the type strain and the two *H. pylori* strains (strain 26695 and J99) for which the genome sequence is available (Tomb *et al.*, 1997; Alm *et al.*, 1998) were compared. Pair-wise and multiple sequence alignments were preformed using ClustalW version 1.7 (data not shown). Preliminary examination of the multiple alignments showed a high level of identity and similarity at the nucleotide and amino acid level respectively. DNA identity scores for pair-wise alignments ranged from 93-99% and from 94-100% using the corresponding similarity scores for amino acid data. Nucleotide and amino acid substitutions occurred at very few sites, indicating that the gene is conserved at the nucleotide level.

6.4.3 Sequence analysis of the HP1085 gene

The HP1085 gene was examined for features associated with a gene encoding an exported protein. Based on a Kyte-Doolittle plot, a hypothetical organisation of the gene product can be deduced (Figure 6.3a). In addition to the N-terminal region corresponding to the predicted signal peptide, there is a region of hydrophobicity at the C-terminal which may be involved in anchoring the protein into a bacterial cell membrane. The signal peptide consists of a positively-charged N domain followed by a hydrophobic stretch (H domain) of 10 residues at the N terminus (Figure 6.3b). The

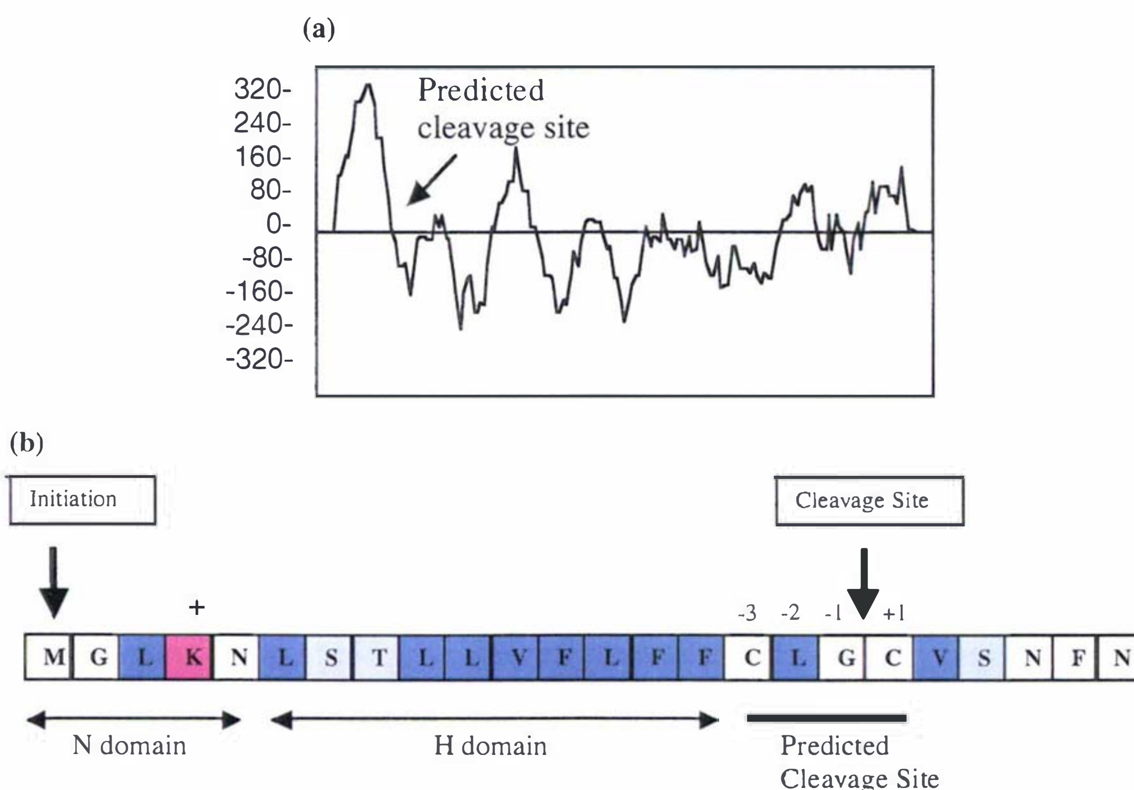


Figure 6.3: (a) Kyte-Doolittle plot of the 18 kDa protein. Hydrophobic regions are shown above the line and hydrophillic below. (b) Signal sequence features of the N-terminal region of the 18 kDa protein showing the predicted domains and cleavage site. hydrophobic polar basic (positively charged)

cleavage site is similar to that of a lipoprotein signal peptidase II (Lsp) cleavage site (CLG-CV) with a cysteine residue at the +1 site (von Heijne, 1990). The predicted signal peptide sequence also has features consistent with lipoprotein signal peptides. The presence of one or more positively charged residues in the N region is universal among bacterial signal peptides, but the length (10 residues) and highly hydrophobic H region is more consistent with lipoprotein signal peptides. In addition, the signal

peptide appears to lack the G or P residue typically found in or around position –6 in signal peptides cleaved by signal peptidase I (LepB) (Pugsley, 1993). The lack of a consensus signal peptidase I cleavage site, commonly A-X-A, also indicates that the signal peptide of this protein is cleaved by a different peptidase. The presence of an aromatic residue at the C terminal is consistent with an outer membrane location.

6.4.4. Expression and purification of the 18 kDa protein

In order to produce the entire 18 kDa protein to high levels, the HP1085 gene was expressed as a β-galactosidase fusion. Upon IPTG induction of *E. coli* XLI-blue cells harboring the pXa-1085 plasmid, a 135 kDa β-gal fusion protein was produced (Figure 6.4). The β-gal-HP1085 fusion protein was purified from the crude lysate by affinity chromatography and the eluted fractions containing the largest proportion of fusion protein, as determined by SDS-PAGE (data not shown) were pooled. A portion of the

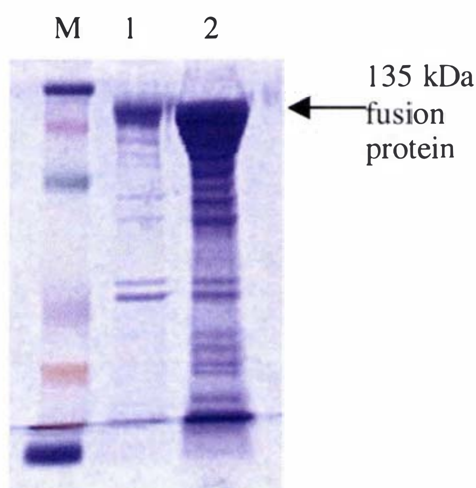


Figure 6.4: Expression of the Beta-galactosidase HP1085 fusion protein. Coomassie stain of lysate protein from an uninduced and induced sample of *E. coli* De3 harbouring pXa1-1085. Cells were induced with 0.2mM IPTG and harvested after 6 hours induction. M, Kaleidoscope marker (Biorad); lane 1, uninduced sample lane 2, induced samples.

purified β-gal fusion protein was cleaved with varying amounts of the protease Factor Xa to determine the optimal conditions to cleave the β-gal portion from the *H. pylori* 18 kDa protein (Figure 6.5 (a)). The β-gal portion of the fusion protein from the entire sample was then removed by proteolytic cleavage with factor Xa (biotin-labelled) at 25°C overnight and was subsequently removed using streptavidin gel. The cleaved β-gal portion was then removed by a second APTG affinity chromatography step yielding pure recombinant protein (Figure 6.5 (b)). The size of the recombinant protein is in

agreement with the calculated molecular mass of the polypeptide deduced from the DNA sequence (approximately 18 kDa). To confirm its identity, the purified 18 kDa recombinant protein was subjected to N-terminal sequencing. The resulting amino acid sequence, GATMEFFTYT, was consistent with the predicted amino acid sequence from the HP1085 ORF.

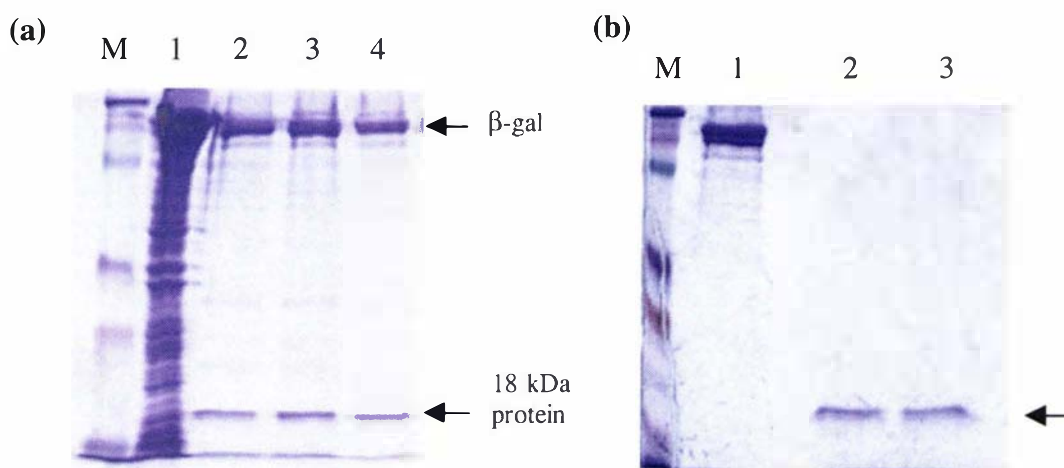


Figure 6.5: Cleavage and purification of the 18 kDa antigen from Beta-galactosidase (β-gal) **(a)** Cleavage of the fusion protein with Factor Xa to release the 18 kDa protein. Lane 1, lysate protein from induced pXa-1085; lane 2,3 and 4, cleavage of the *H. pylori* 18 kDa antigen from β-gal using different ratios of substrate:enzyme **(b)** Pure 18 kDa protein following a second affinity chromatography to remove the cleaved β-gal. Lane 1, *H. pylori*-β-gal fusion protein before cleavage, lanes 2 and 3, released pure 18 kDa antgien. M= Kaleidoscope protein marker (BioRad). Protein was separated on 15% SDS-PAGE gels and stained with Coomassie blue to visualise the protein.

6.4.5 Humoral immune response to the purified 18 kDa antigen

The immunogenicity of the 18 kDa antigen was determined by immunoblotting. Twenty-one serum samples from *H. pylori*-infected patients and eleven non-infected controls were collected and used in individual immunoblot assays to determine the frequency of recognition of the purified protein by antibodies from infected patients (Figure 6.6). Of the 21 *H. pylori* positive samples, the 18 kDa antigen was recognized by 15 sera in Western blots (71%) demonstrating that the protein is immunogenic during the infectious process. Only two of the eleven control patients (18%) recognized the *H. pylori* 18 kDa protein ($p = 0.008$).

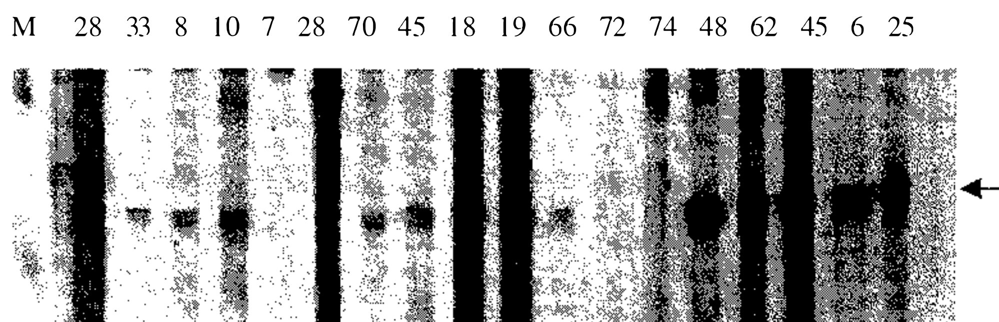


Figure 6.6: Western blot of purified recombinant 18 kDa protein screened with sera from *H. pylori* infected and non-infected individuals. Patient numbers are marked above the corresponding lane. Arrow refers to the position of the 18 kDa antigen. For patient details refer to Table 2.3.

6.4.6 Oral immunisation using recombinant 18 kDa antigen in a mouse model

A mouse model of *H. pylori* infection was used to determine if oral administration of the pure recombinant 18 kDa antigen would induce protective immunity. Sham immunised (PBS + CT) mice (n=6) were used as controls in addition to non-immunised/infected controls (n=4). As a positive control, mice (n=6) were immunised with a *H. pylori* sonicate extract, a regime previously established to protect mice against challenge with *H. pylori* and *H. felis*. Experimental mice (n=6) were immunised with the 18 kDa antigen plus CT as described in section 6.3.7. For each mouse, infection was assessed by determining the number of CFU/g stomach tissue (Figure 6.7) and gastric tissue urease test (Figure 6.8). Mice immunised with the 18 kDa antigen plus cholera toxin showed similar levels of colonisation to both non-immunised (control) and sham immunised mice (PBS). Each mouse in the experimental group (18 kDa) was positive by gastric tissue urease test and showed no significant decrease in CFU/g tissue compared to the control and PBs groups. On the other hand, mice immunised with *H. pylori* sonicate extract (sonicate) showed significantly lower levels of colonisation ($p = 0.0091$) compared to the control and PBS groups, with only 1 out of the 6 mice positive by urease test in this group.

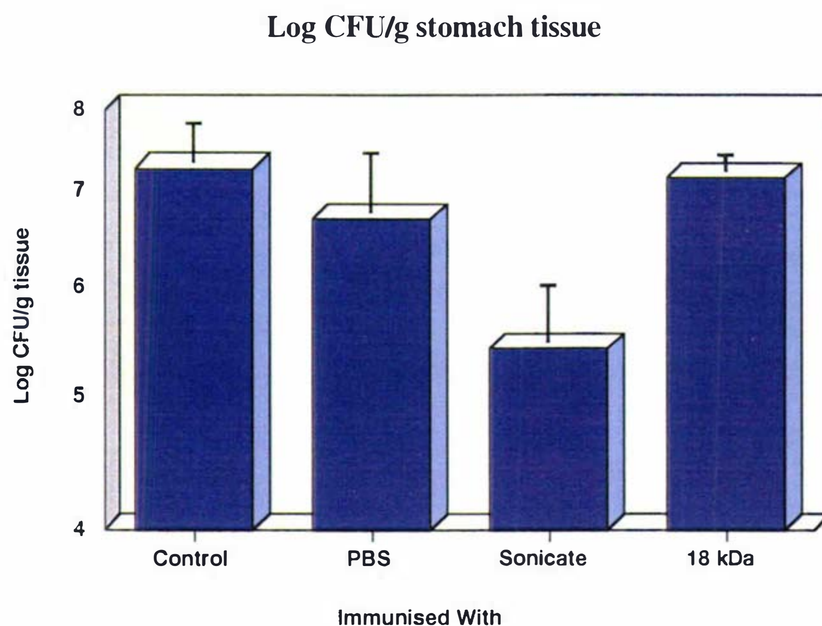


Figure 6.7: Mean log CFU/g stomach of stomach tissue for each group of mice. Mice were either non-immunised (controls) or immunised with PBS, *H. pylori* sonicate or the 18 kDa antigen. Cholera toxin (10 μg) was added as an adjuvant in each immunisation protocol.

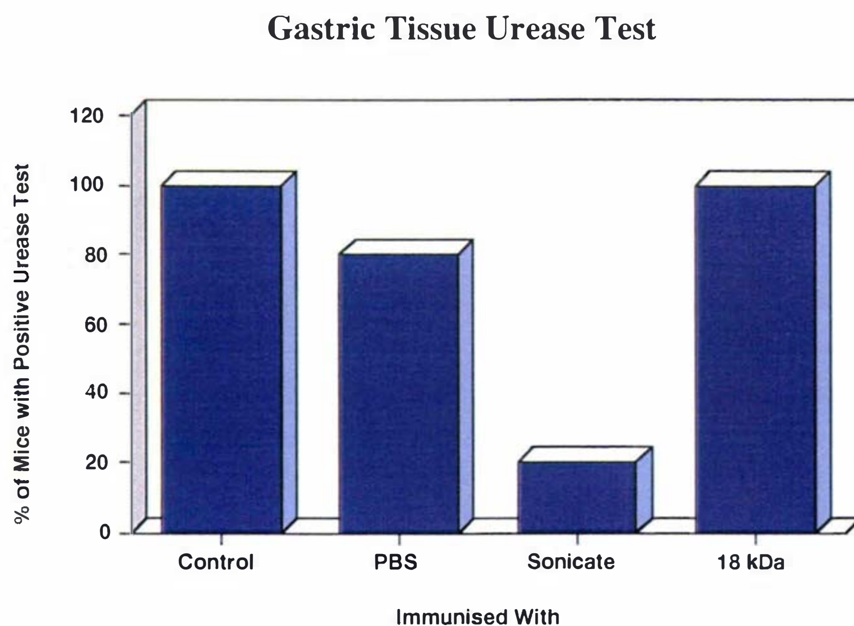


Figure 6.8: Gastric tissue urease test for each group of mice. Stomach tissue was determined as positive or negative as described in section 6.3.6. The number of mice positive for urease acitivty were plotted as a percentage of total number for each group of mice immunised with either PBS, *H. pylori* sonicate or the 18 kDa antigen, in addition to non-immunised controls.

In addition, ELISA results (Table 6.1) for each group illustrated high IgG and IgA antibody titres to both HSPE and cholera toxin (CT) but low antibody titres to the 18 kDa antigen in all groups of mice.

Table 6.1: Antibody titres in mice post-immunisation

Immunised With	No. mice	Faecal IgA to HPSE	Serum IgA to HPSE	Serum IgG to	
				HPSE	18 kDa
PBS + CT	6	0.0620	0.0638	0.7735	0.0333
HPSE + CT	6	0.5943	0.4079	1.3745	0.0309
18 kDa + CT	6	0.0842	0.1190	0.4824	0.2716
Controls	4	0.0954	0.1288	0.6113	ND

PBS = phosphate buffered saline; CT = cholera toxin; HPSE = *H. pylori* sonicate extract.

The results of this preliminary vaccine trial indicated that administration of the 18 kDa protein to mice did not induce protective immunity against infection, under the conditions employed.

6.5 Discussion

One of the challenges to understanding the immune response to *H. pylori* infection is to identify those *H. pylori* proteins that are recognised by the immune cells regulating the host's immune response. Many *H. pylori* surface and secreted proteins are recognised by antibody derived from sera taken from *H. pylori* infected patients (Karczewska *et al*, 1996). In the previous study (chapter 5), screening of *H. pylori*-AP fusion proteins with sera from *H. pylori* infected patients led to the identification of a recombinant fusion protein recognized by patient antibody. Sequencing revealed that the gene encoded an 18 kDa putative outer membrane protein (Tomb *et al*., 1997). The organization of the gene encoding the protein is consistent with an exported protein with regions of hydrophobicity in the C terminal domain that could be involved in membrane anchorage of the protein (Pugsley, 1993). The 18 kDa antigen was designated as a hypothetical

outer membrane protein in the published *H. pylori* genome sequence (Tomb *et al.*, 1997). Support for this comes from the observation that the 18 kDa antigen contains a C-terminal phenylalanine residue, shown to be critical for the efficient sorting to the outer membrane (Struyve *et al.*, 1991). The presence of a potential lipoprotein signal peptide suggests that the 18 kDa antigen may potentially function as a peptidoglycan-associated lipoprotein (PAL). Similar 18 kDa PALs have been found to be immunogenic during infection with other gram-negative bacteria (Spinola *et al.*, 1996; Lim *et al.*, 1997).

Using a combination of Southern blot analysis and PCR, the gene was not detected in *H. felis* or *H. mustelae*. However, since only one strain of each species was screened, the possibility cannot be excluded that the gene may be present in different strains or detected under less stringent conditions used in the Southern blot technique. Nevertheless, these results, and the absence of any homology to sequences in the databases, suggest that the gene may be specific to *H. pylori*. The gene is present in different New Zealand clinical isolates and the geographical diverse types strains CCUG 17874, 26695 (Tomb *et al.*, 1997) and J99 (Alm *et al.*, 1999). A comparison of the DNA sequence of HP1085 from five *H. pylori* isolates and the three type strains showed a variation of up to 3.5% in the DNA sequence suggesting that the gene is highly conserved. This is consistent with the 2 – 5% variation frequently observed when nucleotide sequences of conserved genes from different *H. pylori* isolates are compared. This was observed in the case of the urease genes and the heat-shock protein gene *hsp*B (Suerbaum *et al.*, 1996).

Serology has been used extensively as the basis for diagnostic tests of *H. pylori* infection, and is particularly useful as an initial non-invasive diagnostic tool. Sensitivities of ELISA based methods are generally high, but these assays can be plagued by low specificities due to cross-reactivity (Nilsson *et al.*, 1997). The advantage of the immunoblot technique in serological assays is its accurate detection of antibodies that react with defined antigens from an infectious agent. Immunoblot analysis has therefore been investigated as a more reliable alternative for serodiagnosis in *H. pylori* (Karvar *et al.*, 1997). To investigate the role of the 18 kDa protein in the host's humoral immune response, the purified recombinant protein was used in Western blot assays against a panel of sera from *H. pylori* infected and non-infected individuals.

The majority of infected patients (71%) recognized the protein compared to 16% of non-infected controls.

Although a number of secreted or surface-associated proteins are recognised by infected individuals (Karczewska *et al.*, 1996), there appears to be no correlation between the relative abundance of a particular antigen and its potency as a target for an immunological response (Worst *et al.*, 1996). In addition, recognition of immunodominant antigens during *H. pylori* infection does not often correlate well with disease outcome (Macchia *et al.*, 1993, Jenks *et al.*, 1998). *H. pylori* infected patients produce antibodies against *H. pylori* immunodominant antigens such as the urease B subunit (Worst *et al.*, 1996), LPS (Yokota *et al.*, 1998) and heat shock protein A and B (Suerbaum *et al.*, 1994; Ng *et al.*, 1999), however, recognition of these antigens by antibody from *H. pylori* infected individuals varies from 35% (HspA) to 75% (UreB). To explain this, it has been suggested that genetic restriction in antigen recognition by the host results in individual recognition of some antigens but not others (Worst *et al.*, 1996). Alternatively, the proportion of antibodies in the serum specific for the epitopes in some patients may have been outside the detection limit of the Western blot assay.

The lack of a detectable antibody response to the 18 kDa antigen in some patients may reflect differences in immune response to infection. An alternative explanation is that changing patterns in antigen recognition post infection may have influenced the recognition of the 18 kDa antigen. In previous studies, it was found that immunodominance of individual antigens such as HspA (Perez-Perez *et al.*, 1996) and CagA (Mitchell *et al.*, 1996) changed over the course of infection. The screening criteria employed in this study was designed to detect *H. pylori* antigen consistently recognised by antibody as only one blood sample was taken at one particular point in time. Thus, the lack of an antibody response may reflect the patient's immune status to this antigen at the time the blood was taken. It is also possible that the detection of antibody in only 71% of patients in our study is reflected by an absence of the 18 kDa antigen in some strains of *H. pylori*, as demonstrated with the immunodominant CagA antigen (Cover *et al.*, 1995). However, the evidence presented in our study suggests that the 18 kDa antigen is present in all isolates, irrespective of VacA and CagA status.

One patient diagnosed as negative for *H. pylori* infection, but positive on Western blot, had been diagnosed with an active duodenal ulcer 18 years previously. This result may indicate therefore indicate a long-lasting memory response to a previous infection or exposure to *H. pylori.* However, we cannot exclude the possibility that the initial diagnostic tests used were unable to detect a sub-clinical infection, in the two negative patients containing antibody to the 18 kDa antigen.

The study described in this chapter demonstrated that the 18 kDa *H. pylori* protein is recognized by patient serum IgG antibodies and is therefore expressed *in vivo.* Further investigations were therefore carried out to assess the potential of the 18 kDa antigen to induce a strong IgG response in mice by oral immunization and protect them against *H. pylori* infection. Immunisation with purified recombinant His-tagged 18 kDa antigen did not protect mice against challenge with *H. pylori.* The levels of colonisation, as assessed by CFU counts/g tissue and urease test, were similar for both test mice and controls. Immunisation with a sonicate of *H. pylori* SSI, however, achieved protection levels (80%) comparable to what others have found (Ferrero *et al.*, 1994; Hocking *et al.*, 1999). Although two of the mice in the test group contained low levels of serum IgG antibody to the 18 kDa antigen, this did not correlate with any decrease in colonisation in these animals (data not shown).

A recent study has been carried out (Hocking *et al.,* 1999) in which a number of *H. pylori* recombinant His-tagged antigens administered to mice achieved up to 80% protection from infection following challenge with *H. pylori.* Two antigens, Lpp20 and a 29 kDa protein, were demonstrated to be the most effective protective antigens. Their study used a similar immunisation protocol to that used in the present study, with two notable exceptions. Firstly, Hocking *et al.*, (1999) used specific-pathogen free (SPF) BALB/c mice for the immunisation protocol whereas the mice used in the present study were C57B1/6J, mice that are known to display a stronger cellular infiltrate in the stomach when compared to BALB/c mice (Mohammadi *et al.*, 1996; Ferrero *et al.*, 1998). In addition, the mice were sacrificed only two weeks following challenge. The mice used in this study were challenged with an equal dose of *H. pylori* SSI, but sacrificed 6 weeks following challenge. Despite these differences, it is possible that the failure of the mice to raise a strong antibody response to the 18 kDa antigen contributed to the inability of this antigen to protect against subsequent challenge with *H. pylori.*

The lack of an antibody response in the mice was not due to failure of the immunisation protocol, as it was demonstrated that the mice in each group raised significant antibody to CT. Moreover, the control mice immunised with *H. pylori* sonicate extract were effectively protected against colonisation following challenge with *H. pylori*. This result suggests that the protocol described in this study is an appropriate model for studying antigens that may serve as a basis for the development of a *H. pylori* vaccine and can be used to test other potential candidates.

Chapter 7 Concluding Comments

The aims of the work presented in this thesis were to identify *H. pylori* secreted or surface-associated proteins that might be involved in the immunopathogenesis of disease and to investigate their potential as vaccine candidates. The development of heterologous expression systems has facilitated studies involving the manipulation and analysis of genes from *Helicobacter* species. Expression of genes from a pathogenic bacterium such as *H. pylori* in a non-pathogenic, fast-growing host such as *E. coli*, relies on gene expression signals being recognised in the heterologous gram-negative bacterium. In addition to the structural integrity of the recombinant protein remaining similar, if not identical, to the native form, processing and post-translational modification pathways may also need to be conserved for valid immunological studies. Several immunodominant *H. pylori* proteins have now been expressed in *E. coli* and purified as recombinant proteins. Among these are the urease enzyme (Harris *et al.*, 1998), urease subunits (Ferrero *et al.*, 1994), heat shock proteins (Suerbaum *et al.*, 1994) and the cytotoxin-associated gene product (CagA) (Tummuru *et al.*, 1993). These recombinant proteins display similar antigenicity to the native form of the proteins, with only one exception to date (Manetti *et al.*, 1995).

The development, in our laboratory, of alkaline phosphatase (AP) fusion technology for use with *Helicobacter pylori* has provided a rapid technique to identify *H. pylori* exported proteins (Johnson *et al.*, 1995). Analysis of the deduced amino acid sequences revealed that sixteen fusion proteins producing active AP contained motifs characteristic of bacterial signal peptides. This suggested that the AP-fusion technology was identifying proteins exported by the general secretory pathway (GSP). A number of recombinant plasmids contained cryptic signal peptides that were capable of directing export in *E. coli*, while others contained hybrid genes resulting from non-contiguous DNA fragments cloned into the same plasmid. These latter results demonstrated a limitation associated with this technology. In addition, the difficulty in obtaining full length stable fusion products in *E. coli* was exacerbated by the degradation observed in the majority of the fusion proteins analysed by Western blot. Another limitation is that the strategy identifies only partial gene sequences, which then require locating the full length gene for further characterisation. The bacteriophage

Lambda library was primarily constructed to assist in obtaining full genes for this purpose, however, the release of the *H. pylori* genome sequence in 1997 enabled direct PCR amplification of genes based on the published sequences.

The sequences of the *H. pylori* DNA inserts from twenty-eight recombinant plasmids expressing enzymatically active AP fusion proteins were determined and used to search databases for similarities to known genes. Database searches revealed genes encoding *H. pylori* surface or secreted proteins which had been previously described, including the outer membrane protein (OMP19) gene (*bab*B), which encodes an adhesin involved in binding of *H. pylori* to the human gastric epithelium (Ilver *et al.*, 1998). Homology was also found to the gene encoding a neutrophil activating protein (*nap*A), implicated in the induction of the inflammatory response to *H. pylori* by promoting neutrophil adhesion to the endothelium (Evans *et al.*, 1995). One other clone was shown to contain sequence homology to a *H. pylori* 29 kDa secreted protein (sequence submitted to the gene database). This secreted protein corresponds to the 29 kDa protein which has been shown to induce protective immunity in mice following challenge with *H. pylori* (Hocking *et al.*, 1999). The 29 kDa antigen is currently the focus of a separate study being carried out in our laboratory in collaboration with the Wakefield Gastroenterology Centre, Wellington. Many other sequences represented potentially novel exported protein genes. Further sequencing of clones isolated from the pJEM11 library is likely to identify other putative adhesins or outer membrane components that may contribute to the development of improved diagnosis and control of *H. pylori* infection.

The identification of candidate *H. pylori* antigens for use in recombinant vaccines is a prelude to developing new strategies for combating this infectious disease. Preliminary immunological screening of ten *H. pylori* enzymatically active AP fusion proteins resulted in the identification of a partial 18 kDa protein recognised by sera taken from patients infected with *H. pylori.* On the basis of motifs found within the deduced amino acid structure, the 18 kDa antigen was predicted to be located in the outer membrane. The N-terminal sequence fused to alkaline phosphatase resulted in this fusion protein, and others, being exported to the periplasmic space in *E. coli.* This demonstrated functional recognition of *H. pylori* signal peptides in *E. coli.* However, a conclusion cannot be drawn as to the subcellular location of the 18 kDa protein in *H. pylori* and

future localisation studies would need to be carried out in its natural host. The function of the 18 kDa protein in *H. pylori* is also currently unknown. The creation of null mutants in conjunction with complementation studies would contribute to an understanding of the role this antigen plays in the organism's survival *in vitro* and perhaps *in vivo.*

The gene encoding the 18 kDa protein was cloned and expressed as a recombinant fusion protein for large scale purification of the entire protein. Additional serological screening of the recombinant 18 kDa protein revealed that 71% of *H. pylori* infected individuals contained antibody to this protein, in contrast to only 16% of non-infected individuals (Oliaro *et al.*, in press). Oral administration of the recombinant 18 kDa antigen did not protect mice from infection following challenge with *H. pylori.* In addition, antibody responses in mice directed to this antigen were low or absent in mice, indicating that the antigen did not elicit a strong humoral immune response to the 18 kDa protein. There is evidence to suggest that IgG may play a role in protective immunity against *Helicobacter* infection (Ferrero *et al.*, 1997; Keenan *et al.*, submitted for publication) and therefore, the lack of protection observed in this study may reflect an absence of protective antibody in these mice. It would be interesting to establish if mice chronically infected with *H. pylori* develop an natural antibody response to the 18 kDa antigen. In order to develop strategies for generating the appropriate immune response necessary to induce protective immunity, future research into *Helicobacter* will require a clearer understanding of the immune response that correlates with protection. Other future developments in *Helicobacter* vaccination protocols are likely to include the generation of new mucosal adjuvants without gastrointestinal toxicity and testing of combinations of different cocktail antigens to direct immune responses against different stages of the disease. Alternatively, different routes of administrations and vaccine protocols that do not require a mucosal adjuvant, for example, antigen expression by live carriers, may also be desirable.

In preliminary studies, the work presented in Chapter 3 described an initial characteristion of the human serological response to *H. pylori* antigens and the construction of an *H. pylori* expression library in bacteriophage Lambda. A collaborative project was initiated in which the expression library was used to identify the gene encoding a potential *H. pylori* vaccine candidate (identified as part of an

independent study). The expression library was screened with a monoclonal antibody raised against an immunogenic component of *H. pylori* outer membrane vesicles (OMVs) (Keenan *et al.*, 1998). Subsequent expression of the gene product, detected by this antibody, resulted in the identification of Lpp20 (Kostrzynska *et al.*, 1994). When used in vaccine trials, the *H. pylori* OMV preparation was shown to protect mice against infection following challenge with *H. pylori* (Keenan *et al.* submitted for publication). Moreover, this protection correlated with serum IgG antibody reactivity to the OMV antigen identified as Lpp20. In other studies, outer membrane vesicles have been shown to play a role in pathogenesis (Keenan *et al.*, in press) and it has been suggested that they may function as a delivery system for virulence factors of *H. pylori* (Fiocca *et al.*, 1999). *H. pylori* OMVs are strongly antigenic, and contain proteins, such as Lpp20, with protective properties. Since OMVs from a number of gram negative pathogens can be integrated into the outer membrane of common vaccine strains such as *Salmonella typhimurium aro*A (Kadurugamuwa and Beveridge, 1999), it may be possible to use oral, attenuated vaccine strains containing incorporated *H. pylori* OMVs as multiple epitope vaccine candidates against *H. pylori.*

The study presented in this thesis has contributed to the general body of information concerning the expression and export of *H. pylori* proteins. The number of clones found to express active alkaline phosphatase in *E. coli* indicates that a large number of *H. pylori* exported proteins still remain to be evaluated for their antigenic activity and assessed for their potential as candidates in future vaccine studies.

APPENDIX A: Raw Data for Enzyme Assays in Chapter 5

A1: Alkaline Phosphatase Enzyme Assays (Table 5.1)

Sample	Culture Volume (ml)	OD_{600}	Assay Time (min)	Volume assayed (ml)	Mean OD_{420}	Activity (U/min/ml)
pJHp1	40	0.82	40	0.1	0.263	80.2
pJHp2(a)	40	0.85	40	0.1	0.572	168.2
pJHp2 (b)	40	0.91	40	0.1	0.631	173.4
pJHp4	40	0.80	40	0.1	0.795	248.4
pJHp5	40	0.85	40	0.1	0.662	206.9
pJHp6 (a)	40	0.86	40	0.1	0.530	154.1
pJHp6 (b)	40	0.90	40	0.1	0.400	111.1
pJHp7 (a)	40	0.85	40	0.1	0.170	50.0
pJHp7 (b)	40	0.80	40	0.1	0.110	34.4
pJHp9	40	0.75	40	0.1	0.860	286.7
pJHp10 (a)	40	0.73	40	0.1	0.430	147.2
pJHp10 (b)	40	0.82	40	0.1	0.306	93.3
pJEM	40	0.94	40	0.1	0.023	6.1
pJEM	40	0.80	40	0.1	0.013	4.1
De3	40	0.91	40	0.1	0.020	5.5
De3	40	0.80	40	0.1	0.020	6.2
pJHp W	40	0.90	40	0.1	0.043	11.9
pJHp W	40	0.80	40	0.1	0.019	5.9

Mean OD_{420} = mean absorbance at 420 nm for triplicate wells. Alkaline phosphatase activity was measured as described in Chapter 5.3.2. The results from two separate preparations (a + b) are shown for some samples. pJEM = vector control (no insert); De3 = host strain control (no plasmid); pJHp W = white recombinant pJHp clone.

A2: Enzyme Assays on Cellular Fractions from pJHp20 (Table 5.3)

Sample	OD_{600}	mg/ml	Assayed (10μg)	OD_{420}		Specific Activity*		% Total Activity	
				β-gal	AP	β-gal	AP	β-gal	AP
periplasmic	1.0	0.7	14.2 μl	0.136	1.056	2.74	2.65	20	99.9
intracellular	1.0	1.4	7.1 μl	0.543	0.008	10.92	0.02	80	0.01
periplasmic	1.2	1.0	10 μl	0.286	0.736	14.72	1.84	50	99.9
intracellular	1.2	2.4	4.0 μl	0.700	0.002	14.58	0.01	50	0.01

*Specific Activity = units/ml/min/mg; β-gal = beta-galactosidase; AP = alkaline phosphatase

A3: Enzyme Assays on Cellular Fraction from pJHp clones (Table 5.2)

Sample (cell fraction)	Alkaline Phosphatase			β-galactosidase		
	OD420 average	**Activity**	**%Total Activity**	**OD420 average (min)**	**Activity**	**%Total Activity**
pJHp1 (per)	0.631	157.8	99.3			
pJHp1 (int)	0.004	1.0	0.7			
pJHp1 (ext)	0.016(o/n)	<1	<1			
pJHp2 (per)	0.572	143.0	90.8			
pJHp2 (int)	0.058	14.5	89.2			
pJHp2 (ext)	0.014(o/n)	<1	<1			
pJHp4 (per)	0.795	198.8	94.1			
pJHp4 (int)	0.050	12.5	5.9			
pJHp4 (ext)	0.012(o/n)	<1	<1			
pJHp5 (per)	0.362	90.5	90.0			
pJHp5 (int)	0.042	10.5	10.0			
pJHp5 (ext)	0.014(o/n)	<1	<1			
pJHp6 (per)	0.53	132.5	96.4	0.124 (30)	20.6	1.9
pJHp6 (int)	0.02	5.0	3.6	0.236 (5)	1048.9	98.1
pJHp7 (per)	0.17	42.5	89.5	0.207 (15)	61.3	5.7
pJHp7 (int)	0.02	5.0	10.5	0.274 (6)	1014.8	94.35
pJHp9 (per)	0.860	165.0	95.7	0.160 (40)	17.8	3.4
pJHp9 (int)	0.030	7.5	4.3	0.230 (10)	511.1	96.6
pJHp10 (per)	0.42	105.0	89.4	0.180 (40)	20.0	3.3
pJHp10 (int)	0.05	12.5	10.6	0.260 (10)	577.8	96.7
pJEM (per)	0.023	5.75	N/A	0.188 (15)	55.7	12.8
pJem (int)	0.010	2.50	N/A	0.170 (10)	377.8	87.2
pJem (ext)	0.008(o/n)	<1	N/A	ND	ND	ND
pJHp W (per)	0.043	10.75	N/A	0.170 (15)	50.4	12.3
pJHp W (int)	0.024	6.0	N/A	0.162 (6)	360.0	87.7
pJHp W (ext)	0.010(o/n)	<1	N/A	ND	ND	ND
De3 (per)	0.020	5.0	N/A	0.22 (40)	24.4	1.1
De3 (int)	0.011	2.75	N/A	0.491 (5)	2177.8	98.9
De3 (ext)	0.008(o/n)	<1	N/A	ND	ND	ND

Per = periplasmic space protein fraction; int = intracellular protein fraction; ext = extracellular protein fraction; N/A = not applicable; ND = not done.
For the β–galactosidase assay, the assay time varied with different samples and is therefore recorded in brackets. One hundred microlitres of each sample was assayed for alkaline phosphatase under standard conditions for 40 mins. For the β-galactosidase assay, 50 ul of periplasmic and 10 μl of intracellular protein fractions were assayed.

APPENDIX B: Raw Data for Mouse Immunisation Trial in Chapter 6

Mouse No.	Sex	Imm. with	Serum IgA to HPSE	Serum IgG to HPSE	Serum IgA to CT	Serum IgG to CT	Serum IgG to 18kDa	UT	CFU/g tissue
19	F	PBS	0.0440	0.6980	0.3810	1.4100	0.0265	+	5.32E+06
20	F	PBS	0.0300	0.1915	0.2015	1.2070	0.0470	-	1.37E+05
21	F	PBS	0.2260	1.6660	1.6480	1.1380	0.0525	+	1.54E+07
22	F	PBS	0.0225	0.5775	0.9515	1.4830	0.0345	+	1.13E+07
23	F	PBS	0.0345	1.1190	0.7420	1.5740	0.0175	+	1.01E+07
24	M	PBS	0.0255	0.3890	0.8105	1.3400	0.0220	+	7.92E+06
25	F	HPSE	0.0635	1.8920	0.5435	1.4120	0.0280	-	1.46E+05
26	F	HPSE	0.0795	1.7740	0.4705	1.5940	0.0180	-	6.00E+04
27	F	HPSE	0.5850	1.5800	0.9180	1.4720	0.0150	-	9.30E+04
28	M	HPSE	0.1650	1.5000	0.4330	1.3860	0.0525	-	1.02E+06
29	M	HPSE	0.0625	0.3070	0.8565	1.4590	0.0345	+	1.28E+06
30	M	HPSE	1.4920	1.1940	2.3950	1.7480	0.0375	-	3.66E+05
31	F	18kD	0.1275	1.3660	1.0900	1.8800	0.2495	+	6.34E+06
32	F	18kD	0.2185	0.5980	0.7590	1.8310	1.185	+	6.33E+07
33	F	18kD	0.2175	0.3060	0.6300	1.9890	0.0405	+	2.51E+07
34	F	18kD	0.0850	0.3400	0.3630	1.3260	0.0550	+	1.66E+07
35	F	18kD	0.0255	0.0880	0.4915	1.5670	0.0660	+	2.17E+07
36	F	18kD	0.0400	0.1965	0.5330	1.2510	0.0335	+	1.61E+07

HPSE = *H. pylori* sonicate extract; CT = cholera toxin; UT = urease test; CFU/g tissue = colony forming units per gram of stomach tissue

APPENDIX C: One letter codes for amino acids and deoxyribonucleosides

amino acids: G, A, V, L, I, P, F, Y, W, S, T, C, M, N, Q, D, E, K, R, H for glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, tyrosine, tryptophan, serine, threonine, cystein, methionine, asparagine, glutamine, aspartate, glutamate, lysine, arginine and histidine, respectively.

deoxyribonucleosides: A, C, G, T for deoxyadenylate, deoxycytidylate, deoxyguanylate and deoxythymidylate, respectively.

APPENDIX D: List of Publications related to this thesis

J Keenan, **J. Oliaro**, N. Domigan, H. Potter, G. Aitken and R. Allardyce Immune responsiveness to an 18 kDA outer membrane antigen identifies Lpp20 as an *Helicobacter pylori* vaccine candidate. Submitted for publication.

J. Oliaro, R.D. Johnson, W. Chen, V. Chadwick and A. Murray Identification of an immunogenic 18 kDa protein of *Helicobacter pylori* using alkaline phosphatase gene fusions. *Journal of Medical Microbiology* (In Press)

*R.D.Johnson, **J.Oliaro**, W Chen, V.S. Chadwick and A. Murray (1997) Formyl Met-Leu-Phe peptides and *Helicobacter pylori. Biomedical Letters* **56** 105-110

J. Oliaro, R.D. Johnson, W. Chen, V.S. Chadwick and A. Murray (1997) Identification of *Helicobacter pylori* genes encoding exported proteins. Abstract. In Proceedings of the Australian Society for Biochemistry and Molecular Biology Vol **29** B2-105

R.D. Johnson, **J. Oliaro** and A. Murray. (1995) Identification of *Helicobacter pylori* genes encoding exported proteins. *Biomedical Letters* **51** 267-269

* This publication arose from a separate study which did not form part of this PhD project.

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