Genetic Determinants of *Helicobacter pylori* Vacuolating Cytotoxin Activity

Darren Peter Letley, BSc (Hons)

Published works submitted to the University of Nottingham

for the degree of Doctor of Philosophy

December 2011

CONTENTS

ACKNOWLEDGEMENTS

I would like to begin by thanking Prof John Atherton, who introduced me to the world of *Helicobacter pylori* all those years ago. Your knowledge, enthusiasm, patience and wit have supported the research presented here, and I cannot thank you enough for all the help and guidance you have given me over the years.

Being a long standing member of the Hp Group, I have seen many people come and go, and I've been extremely fortunate to have always worked with great people and made good friends. I would like to say a special thank you to all the research technicians who have contributed to the work presented here, and who have been a joy to work with: Rachel Twells, Brian Dove, Keith Bishop and particularly Jo Rhead, who I have worked with for such a long time, and who has become a dear friend. I would like to thank the PhD students who have been so fun to supervise over the years: Geetha, Rachael, Francisco, Charlotte, Sapna, Emily, Khiyam and Katie. Thanks to all the rest of the past and current members of the Hp Group for keeping me smiling: Ameer, Amin, Andy, Borgel, Christian, Christine, Fei Fei, Géraldine, James B, James H, Jane, Jody, Karen, Katherine, Maher, Marie, Martin, Nawfal, Neil, Nicki, Richard A, Richard I, Rob, Rupert and Zuraihan. You have all made the Nottingham Hp Group such a great team to be a part of. Special thanks also to Alan Cockayne for all his invaluable practical advice and friendship.

Finally I would like to thank my family for all their love, support and endless encouragement, especially my loving partner Jo, without whom I would be lost, and my two boys, George and William, who bring me so much joy.

STATEMENT OF CONTRIBUTION TO PUBLISHED WORKS

I hereby certify that I was the main independent researcher and author on all five of the papers submitted, being responsible for leading each project, the design of all experiments, performing the majority of laboratory work with technical support under my supervision (as detailed below), data analysis and the preparation of manuscripts. All work was performed in the laboratory of Prof. John C. Atherton, the principle investigator and grant holder, and who is therefore senior author on all papers.

Additional author contributions were as follows:

J. L. Rhead is a research technician working under my supervision. With me, she constructed and characterised *vacA* mutants and performed *vacA* sequence analysis for Chapter 6. Her contribution to this part of the paper's experimental design and data analysis are reflected in joint first authorship of this paper. She also provided technical support for Chapters 4 and 5.

M. Mohammadi, M. A. Mohagheghi, and M. E. Hosseini provided genomic DNA from Iranian *H. pylori* clinical strains for Chapter 6, which I typed for *vacA* polymorphisms.

N. Hussein performed *cagA* PCR typing described in Chapter 6.

K. Bishop, R. J. Twells, and B. Dove provided technical support under my supervision on Chapter 5 (KB) and Chapter 4 (RJT and BD).

A. Lastovica, and J. A. Louw provided the *H. pylori* clinical isolates from South Africa studied in Chapter 2.

C. J. Hawkey was a joint grant holder with J. Atherton for funding contributions to Chapter 2.

Signed: Date:

Mr Darren P. Letley, Research Fellow
Nottingham Digestive Diseases Centre, University of Nottingham

I hereby confirm that I agree with the above statement:

Signed: Date:

Mrs Joanne L. Rhead, Research Technician
Nottingham Digestive Diseases Centre, University of Nottingham

I hereby confirm on behalf of myself and all remaining contributing authors that the above statement is correct.

Signed: Date:

Prof John C. Atherton, Head of School of Clinical Sciences
Nottingham Digestive Diseases Centre, University of Nottingham

SUMMARY

Helicobacter pylori **colonises the human gastric mucosa of approximately half the world's population, where it induces a chronic active gastritis and persists lifelong in the absence of medical intervention. Although most infected patients remain asymptomatic, gastric colonisation by** *H. pylori* **is the main cause of peptic ulcer disease and a significant risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. The outcome of** *H. pylori* **infection depends on strain virulence, host response and environmental factors. A major** *H. pylori* **virulence determinant is the vacuolating cytotoxin VacA, which has many effects on mammalian cells, the most characterised being the induction of cytoplasmic vacuolation in epithelial cells. VacA is an autotransporter, and is translated as a 139 kDa polypeptide which undergoes N- and C-terminal processing resulting in a mature, secreted toxin of 89-95 kDa. A single pair of cysteine residues, usually spaced 11 residues apart, are found towards the C-terminal end of the passenger domains of many autotransporters including VacA. We have shown that these cysteine residues are required for high level VacA production (Letley, DP,** *et al.* **2006.** *Microbiol.* **152:1319), which may have implications for the autotransport process.**

All *H. pylori* **strains possess the gene,** *vacA***, encoding the secreted toxin, and most express VacA protein. However, not all strains possess vacuolating activity, and those that do are strongly associated with peptic ulcer disease and gastric adenocarcinoma. Sequence polymorphisms occur throughout** *vacA***, the two most diverse regions being the signal region (s1 or s2) and the mid region (m1 or m2). Type s1 strains may be further differentiated into s1a, s1b and s1c subtypes. Natural mosaicism exists such that all combinations of signal and mid region type exist, including the rare s2/m1 type which we reported for the first time (Letley, DP,** *et al.* **1999.** *J. Clin. Microbiol.* **37:1203). The existence of all possible** *vacA* **allelic combinations supports the proposal that mosaicism results from recombination between different** *vacA* **alleles. Geographical variation in** *vacA* **allelic type exists. s1a and s1b subtypes are common in the USA and Europe, Asian strains are commonly s1c-type and we have found the s1b-allelic type to be predominant in South Africa (Letley, DP,** *et al.* **1999.** *J. Clin. Microbiol.* **37:1203). s1b strains are less strongly associated with disease than s1a strains in the west, which may have implications for the level of** *H. pylori***-related disease in Africa.**

Functionally, s1 strains are associated with vacuolating activity *in vitro* while s2 VacA is invariably nonvacuolating. Using isogenic *vacA* chimeric and site-directed mutagenesis approaches we have shown that a 12 amino acid hydrophilic N-terminal extension specific to the s2-form blocks vacuolating activity (Letley, DP, and JO Atherton. 2000. *J. Bactelol.* **182:**3278; Letley, DP, *et al.* 2003. *J. Biol. Chem.* **278:**26734). Signal region type also determines toxin production through differences in the promoter region. Natural polymorphism within the mid-region determines the cell specificity of vacuolation, such that m1 forms vacuolate a wider range of epithelial cell lines than m2 (Letley, DP *et al.* 2003. *J. Biol. Chem.* **278:**26734-41). Exchange mutagenesis experiments led to the discovery of a new determinant of toxin activity, the *vacA* intermediate region (i1 or i2) (Rhead, JL, DP Letley, *et al. Gastroenterol.* **133:**926). While s1/m1 and s2/m2 strains were invariably i1 and i2 types respectively, s1/m2-type strains varied in i-region type, and this correlated with their vacuolating activity *in vitro*. An isogenic mutagenesis approach confirmed that i-type determined vacuolating activity. A simple POR-based i-region typing system was devised and used to show that i1-type strains were significantly associated with gastric adenocarcinoma among a sample of 73 Iranian strains. Furthermore, i-region type was a better marker of disease-associated *H. pylol* strains than previously studied signal and mid region types.

CHAPTER 1
INTRODUCTION

1.1 *Helicobacter pylori* history and microbiology

For most of the 20th Century the mammalian stomach was generally regarded as being free from bacterial infection owing to its acidic nature, despite early observations of spiral-shaped bacteria in the stomachs of dogs (Bizzozero, 1893) and humans (Krienitz, 1906). The successful culture of curved bacteria, which had been observed in human gastric biopsies from patients with gastritis and peptic ulceration during histological examination, led to the initial identification of a novel 'pyloric' Campylobacter species associated with gastric disease (Marshall and Warren, 1984; Warren and Marshall, 1983). The general acceptance that chronic active gastritis and peptic ulcer disease were mainly attributable to a bacterial infection followed the fulfilment of Koch's postulates: self-infection experiments demonstrating that these curved bacteria isolated from gastric tissue were capable of inducing a chronic, active gastritis, which could be successfully treated by antibiotic therapy (Marshall *et al.*, 1985; Morris and Nicholson, 1987). Morphological and biochemical characterisation of this newly described *Campylobacter pylori* species showed that the organism was sufficiently different to other members of the *Campylobacter* genus (Goodwin *et al.*, 1985), and it was reclassified under a new genus as *Helicobacter pylori* (Goodwin *et al.*, 1989).

***H. pylori* is a Gram-negative bacterium with a curved rod morphology (reviewed in Jenks, 2006; Kusters *et al.*, 2006). It is a fastidious and microaerophilic microorganism, which requires a humid atmosphere of 2–5% O_2 and 5–10% CO_2 at 37°C for optimal laboratory growth. *H. pylori* can be cultured on a variety of solid base media supplemented with 5–10% horse or sheep blood, or in liquid broth (brain-heart infusion, Brucella or Isosensitest) supplemented with either 5–10% foetal calf serum or 0.2–1% B-cyclodextrin. The bacteria can typically be isolated from gastric biopsies in 3-5 days, forming smooth, translucent colonies of approximately 1 mm diameter. Prolonged culture of *H. pylori* results in an irreversible conversion to a nonculturable, coccoid form, the significance of which remains unclear despite extensive study. *H. pylori* is catalase, oxidase and urease positive, the latter reaction being characteristically rapid and most frequently used for initial identification, and is motile, possessing 4-8 sheathed, unipolar flagella.**

1.2 *H. pylori* epidemiology and associated disease

In the absence of antibiotic therapy, *H. pylori* colonisation of the human stomach persists for many decades, resulting in chronic active gastritis characterised by infiltration of neutrophils and mononuclear cells into the gastric mucosa (Marshall and Warren, 1984; Marshall *et al.*, 1985; Morris and Nicholson, 1987; Morris *et al.*, 1991; Warren and Marshall, 1983). It is generally accepted that *H. pylori* is acquired in early childhood, and since there has been no identifiable animal vector, transmission is thought to most likely involve human-to-human contact through oral-oral or fecal-oral routes from close family members. The prevalence of infection correlates inversely with socioeconomic status, and worldwide infection rates vary from more than 80% in developing countries to less than 40% in the developed world (reviewed in Kusters *et al.*, 2006; Malaty, 2007). Furthermore, *H. pylori* prevalence is declining owing to factors such as increased antimicrobial use, improved hygiene and smaller family groups. While the vast majority of cases of *H. pylori* infection remain asymptomatic, persistent colonisation of the gastric mucosa by this bacterium has been associated with several severe pathological outcomes, and consequently *H. pylori* is regarded as an important gastric pathogen.

1.2.1 Acute and chronic gastritis

A human volunteer study has shown that the acute phase of *H. pylori*-associated gastritis is characterised by mild to moderate dyspeptic symptoms, including abdominal cramps, nausea and a feeling of fullness, peaking 9-12 days post infection (Graham *et al.*, 2004). These symptoms resolved within a month of infection. A state of hypochlorhydria was induced following infection, lasting for several months. Histological examination of biopsies taken 2 weeks post-infection revealed both acute and chronic inflammatory cells had infiltrated the gastric mucosa, indicating a rapid onset to a chronic pattern of gastritis. *H. pylori*-associated chronic gastritis is characterised by the presence of organised mucosal lymphoid follicles (Eidt and Stolte, 1993; Genta *et al.*, 1993; Zaitoun, 1995). Where gastric acid production is maintained, persistent *H. pylori* colonisation is mainly restricted to the antrum resulting in an antral-predominant gastritis. Low acid production is associated with colonisation of both the antrum and corpus resulting in a pan-gastritis, where inflammatory factors may then further

suppress parietal cell function enhancing the hypochlorhydria (Kusters *et al.*, 2006). Despite active inflammation of the gastric mucosa, *H. pylori*-associated chronic gastritis is often asymptomatic. Persistent *H. pylori*-induced gastritis may eventually lead to atrophic changes in the normal mucosal structure, such as the loss of gastric glands, and the development of intestinal metaplasia.

1.2.2 Peptic ulcer disease

In developed countries, about 10-15% of *H. pylori*-infected individuals develop peptic ulcer disease (PUD) (reviewed by Kuipers *et al.*, 1995; Kusters *et al.*, 2006). Initial studies soon after the discovery of *H. pylori* found that persistent gastric colonisation by this bacterium accounted for approximately 95% and 85% of duodenal and gastric ulcer cases, respectively, the remainder being due, almost entirely, to aspirin and non-steroidal anti-inflammatory drug use. The prevention of ulcer reccurrence through *H. pylori* eradication, and the declining prevalence of *H. pylori* means that the proportion of PUD cases attributable to *H. pylori* has declined. Peptic ulceration is most common at mucosal sites where inflammation is severe, and is strongly influenced by the level of gastric acid production (Van Zanten *et al.*, 1999). In patients with hyperchlorhydria and antral predominant *H. pylori* colonisation, ulcers are mainly found in the duodenal bulb, the area of the duodenum most exposed to gastric acid, and in the antroduodenal transition zone. In contrast, patients with reduced acid production and a resulting pangastritis, have more severe inflammation at the junction between the corpus and antrum, the most common site of gastric ulcers. *H. pylori*-infected individuals with intermediate acid secretion are less likely to develop PUD. In the west, duodenal ulceration is more common than gastric ulceration, while the reverse is observed for developing countries. These differences in PUD epidemiology may be attributable to differences in nutrition and hygiene levels affecting the levels of acid production in populations (Van Zanten *et al.*, 1999). Gastric ulceration occurs more frequently in older adults, while duodenal ulcers are most common in the 20-50 age group. The reasons for this are unknown.

1.2.3 Gastric adenocarcinoma

Gastric adenocarcinomas are neoplastic lesions which initiate in the glandular cells of the mucosa and grow to invade further layers of the gastric wall (muscularis mucosae, submucosa and muscularis externa). These tumours account for about 90-95% of all gastric malignancies (Shang and Peña, 2005). Gastric adenocarcinomas may be located within the cardia, the region of the stomach which borders the oesophagus (proximal-type), or elsewhere in the stomach (non-cardia or distal-type), and may be well differentiated (intestinal-type), or undifferentiated (diffuse-type) (reviewed in Fox and Wang, 2007). *H. pylori* infection is a significant risk factor for non-cardia gastric adenocarcinoma (Parsonnet *et al.*, 1991a; Parsonnet *et al.*, 1991b), and as such *H. pylori* has been classified as a class I carcinogen by the World Health Organisation. In the west, *H. pylori*-infected patients have an estimated 1-2% lifetime risk of developing gastric cancer, which equates to 60-80% of gastric cancer cases in the developed world being attributed to persistent *H. pylori* colonisation (Kusters *et al.*, 2006). Although the incidence of gastric cancer in western countries is slowly declining with the decreasing prevalence of *H. pylori* infection, gastric cancer is still the fourth most common cancer worldwide, and its ranking as the second biggest cause of cancer-related death means that it is a major public health concern, especially in areas of very high incidence such as some regions of East Asia.

Like gastric ulceration, the development of gastric cancer in *H. pylori*-infected patients is associated with pan-gastritis and reduced acid secretion. Individuals with this pattern of *H. pylori* infection develop atrophic gastritis and intestinal metaplasia more quickly than patients with an antral-predominant infection, and these pathological outcomes increase the risk of intestinal-type gastric adenocarcinoma (reviewed in Fox and Wang, 2007; Kusters *et al.*, 2006). Correa (1988) proposed a pathway describing the histological changes leading to intestinal-type gastric adenocarcinoma beginning with superficial gastritis followed by chronic inflammation, atrophic gastritis, intestinal metaplasia, dysplasia and finally carcinoma. *H. pylori* infection clearly plays a role at the beginning of this pathway establishing chronic gastritis. However, studies have shown that while eradication of *H. pylori* can lessen atrophy and intestinal metaplasia, cancer risk is only reduced in patients without these precancerous

changes (Leung *et al.*, 2004; Mera *et al.*, 2005; Wong *et al.*, 2004), suggesting that *H. pylori* is not a major contributor to the latter stages of the Correa pathway.

1.2.4 Mucosa-associated lymphoid tissue (MALT) lymphoma

In addition to neoplasia arising in the gastric epithelium, *H. pylori* infection is also associated with malignancy of the gastric mucosa-associated lymphoid tissue (MALT)(Parsonnet *et al.*, 1994; Wotherspoon *et al.*, 1991). While not normally found in the stomach, MALT is often present in the gastric mucosa of *H. pylori*-infected patients. In less than 1% of *H. pylori*-infected individuals, a monoclonal population of lymphocytes, usually B cells, may proliferate to form a MALT lymphoma, and about 90-95% of cases of this malignancy can be attributed to *H. pylori*. Gastric MALT lymphomas are the most common form of extranodal non-Hodgkin lymphoma, and represent about 7% of all gastric tumours (reviewed in Morgner *et al.*, 2007). It has been suggested that the neutrophilic response induced by *H. pylori,* results in the release of reactive oxygen species which could generate the genetic abnormalities in active B cells required for MALT lymphoma development (Morgner *et al.*, 2007). Gastric MALT lymphoma often responds well to *H. pylori* eradication therapy, with complete remission being reported in about 80% of cases, emphasising the central causative role of this pathogen in the disease process (Cavanna *et al.*, 2008; Morgner *et al.*, 2007). Long term outcome following *H. pylori* eradication is often favourable, with most patients staying in complete remission for at least 5 years (Chen *et al.*, 2005). Thus, *H. pylori* eradication therapy is the preferred first line treatment for low grade MALT lymphoma in *H. pylori*-positive patients.

1.2.5 Gastro-oesophageal reflux disease

Gastro-oesophageal reflux disease (GORD) occurs when the acidic contents of the stomach frequently pass into the oesophagus, and several processes contributing to GORD have been described including inefficient constriction of the gastro-oesophageal sphincter, delayed gastric emptying, reduced oesophageal motility, insufficient mucosal defence, hiatus hernia and the toxic action of acid and bile (McNamara and O'Morain, 1999). The most common symptoms are frequent episodes of heartburn and acid regurgitation, although many GORD patients present with other symptoms, and it is estimated that 21-44% of the adult population

have frequent GORD symptoms (McNamara and O'Morain, 1999). Complications of severe GORD include oesophagitis (affecting about 2% of adults), carditis, Barrett's oesophagus (intestinal metaplasia and dysplasia affecting the oesophageal epithelium) and carcinomas of both the cardia and oesophagus.

Many studies have found that *H. pylori* infection does not contribute to the development of GORD, as the prevalence of *H. pylori* is not significantly higher in GORD patients than controls (reviewed in McNamara and O'Morain, 1999). On the contrary, it has been suggested that *H. pylori* colonisation may actually protect against GORD by reducing gastric acidity. This is supported by several lines of evidence: some studies have found that *H. pylori* prevalence is lower in GORD patients, and that *H. pylori*-infected GORD patients are more likely to harbour less virulent strains; the incidence of GORD and oesophageal adenocarcinoma is increasing despite the declining prevalence of *H. pylori* infection; and there are some reports of an increase in GORD incidence following *H. pylori* eradication (Kusters *et al.*, 2006; McNamara and O'Morain, 1999).

1.2.6 Treatment of *H. pylori* infection

H. pylori is sensitive to a variety of antibiotics *in vitro*, but the efficacy of single drug therapy is limited by the localisation of the bacterium within the low pH, viscous environment of the gastric mucus layer. The most commonly used eradication treatment is a triple therapy combining two antibiotics with either a proton pump inhibitor (PPI) or a bismuth compound. Quadruple therapies, using both PPI and bismuth in combination with two antibiotics, have also been used effectively. *H. pylori* is sensitive to bismuth compounds, although the mechanism of action is unknown. The most commonly used antibiotics are tetracycline, amoxicillin, imidazoles such as metronidazole, and macrolides such as clarithromycin (Kusters *et al.*, 2006). Using these treatment strategies, eradication rates over 80% are common, although these rates have decreased more recently (Egan *et al.*, 2007). A major cause of eradication failure is the presence of antibiotic resistant strains, the most commonly affected drugs being metronidazole and clarithromycin. New effective treatment strategies include sequential therapy (PPI and amoxicillin for 5 days immediately followed by PPI, clarithromycin and tinidazole for 5 days) and the use of fluoroquinolone antibiotics such as

Levofloxacin in a triple therapy regime (Egan *et al.*, 2007). There has also been a lot of interest in the use of vaccination to both prevent and eradicate *H. pylori* infection. Although studies using animal models have shown that a Th2 polarised immune response to a *H. pylori* vaccine can be an effective treatment strategy, limited vaccination trials in humans, mainly using recombinant urease, have so far been unsatisfactory (reviewed by Kabir, 2007).

1.3 *H. pylori* colonisation and virulence factors

Research on factors determining the outcome of *H. pylori* infection have focused on host characteristics, particularly the immune response, environmental factors such as smoking, diet and the use of non-steroidal anti-inflammatory drugs, and the virulence of the infecting strain. Several *H. pylori* virulence determinants have been described, the two most important and extensively studied factors being the vacuolating cytotoxin VacA (discussed in Sections 1.4-1.7) and the cytotoxin-associated gene (*cag*) pathogenicity island. In addition to these major virulence determinants, *H. pylori* produces other proteins which are essential for colonisation of the human gastric mucosa, or are useful markers of disease risk.

1.3.1 *cag* pathogenicity island

Soon after the discovery that broth culture supernatant from some strains of *H. pylori* could induce cytoplasmic vacuolation *in vitro*, two supernatant proteins of size 82 kDa and 128 kDa were identified as being associated with this cytotoxic activity, and were immunoreactive with human sera (Cover *et al.*, 1990). The 82 kDa protein was later identified as the vacuolating cytotoxin (Cover and Blaser, 1992), while the gene encoding the 128 kDa protein was cloned and named the cytotoxin associated gene, *cagA* (Tummuru *et al.*, 1993). It was later shown that *cagA* was located at one end of a 37-40 kb pathogenicity island (PAI) containing 27-31 genes (Akopyants *et al.*, 1998; Censini *et al.*, 1996). In Western countries, about 60% of strains possess the *cag* PAI, and its presence has been associated with more intense inflammation, and higher rates of peptic ulcer disease and gastric adenocarcinoma (reviewed in Kusters *et al.*, 2006; Peek and Crabtree, 2006). Such associations have not been observed in many Far Eastern populations because virtually all *H. pylori* strains isolated from these

regions possess the *cag* PAI. However, it is interesting to note that such regions often have a high prevalence of gastric adenocarcinoma.

The pathogenic mechanisms by which CagA contributes to *H. pylori*-related disease have been the subject of extensive study. The *cag* PAI has been shown to encode a type IV secretion system which delivers CagA through a syringe-like structure directly into the cytoplasm of host cells (Backert *et al.*, 2000; Odenbreit *et al.*, 2000), where it becomes phosphorylated on the tyrosine residues of EPIYA motifs by host Src family kinases (SFKs) (Asahi *et al.*, 2000; Segal *et al.*, 1999; Selbach *et al.*, 2002; Stein *et al.*, 2000; Stein *et al.*, 2002). The number of EPIYA motifs present near the C-terminal end of CagA varies between strains, and this positively correlates with the level of CagA phosphorylation, the degree of CagA-induced cytoskeletal changes observed in epithelial cells when co-cultured with *H. pylori* (the so called 'Hummingbird' phenotype characterised by cellular elongation and protrusions), and gastric adenocarcinoma (Argent *et al.*, 2004a; Azuma *et al.*, 2002; Higashi *et al.*, 2002a; Segal *et al.*, 1999; Yamaoka *et al.*, 1998). Phosphorylated CagA interacts with the tyrosine phosphatase SHP2, an important regulator of growth factor signalling (Higashi *et al.*, 2002b). In fact it has recently been shown that CagA mimics the function of the normal activator of SHP2, the eukaryotic scaffold adaptor Grb-associated binder (Gab) protein (Botham *et al.*, 2008). Deregulation of SHP2 signalling and subsequent inactivation of focal adhesion kinase (FAK) and prolonged activation of Erk MAP kinase leads to the CagA-dependent morphological changes associated with 'hummingbird' phenotype (Higashi *et al.*, 2004; Higuchi *et al.*, 2004; Tsutsumi *et al.*, 2006). CagA-dependent prolonged activation of Erk MAP kinase may also be a mechanism for deregulating proliferation (Hatakeyama, 2006). Interestingly, phosphorylated CagA is able to attenuate its biological effects by activating C-terminal Src kinase (Csk), which in turn inactivates SFKs preventing further phosphorylation of CagA (Tsutsumi *et al.*, 2003). The inactivation of SFKs, together with competition from excess CagA, affects the phosphorylation of natural SFK substrates. The reduced phosphorylation of one such substrate, cortactin, may also contribute to the formation of 'hummingbird' phenotype owing to its role in regulating actin rearrangement (Selbach *et al.*, 2003).

Studies have also reported biological effects of translocated CagA which are not dependent on its phosphorylation. These include interaction with the proto-oncogenic hepatocyte growth factor receptor c-Met, stimulating cell proliferation and motility (Churin *et al.*, 2003); activation of the Ras-MAP kinase pathway via interaction with Grb2, promoting gastric epithelial cell growth, motility and scattering (Mimuro *et al.*, 2002), and activation of nuclear factor kappa B (NFκB), leading to IL-8 induction (Brandt *et al.*, 2005); disruption of the gastric epithelial cell apical-junctional complex affecting cell-cell adhesion and resulting in an invasive phenotype (Amieva *et al.*, 2003; Bagnoli *et al.*, 2005); and activation of the nuclear factor of activated T-cells (NFAT) transcription factor family in gastric epithelial cells via stimulation of the calcium-dependent serine/threonine phosphatase calcineurin, with subsequent induction of NFAT-regulated gene expression (Yokoyama *et al.*, 2005). In addition to these biological effects of CagA translocation, contact between the *cag* type IV secretion machinery and the host epithelial cell leads to the increased expression of proinflammatory cytokines such as IL-8. It has been shown that the *cag*-dependent leakage of peptidoglycan into host cells signals this proinflammatory response through its recognition by Nod1, resulting in the activation of NFκB (Viala *et al.*, 2004).

Genotypic analysis of *H. pylori* clinical isolates from around the world has shown that the majority of *cag* PAI positive strains also possess the *vacA* type s1 allele associated with vacuolating activity (see Section 1.7), while *cag* PAI negative strains usually possess the non-vacuolating s2 allelic form of *vacA*. This association is not due to clonality, or genetic linkage given their distant locations on the *H. pylori* chromosome. A simple functional dependence is not responsible either, as inactivation of CagA does not abolish vacuolating activity (Tummuru *et al.*, 1994), and a *vacA* null mutant strain is still able to deliver CagA into AGS cells inducing a hummingbird phenotype and IL-8 secretion (Argent *et al.*, 2008). However, mutagenesis studies suggest that CagA and VacA may down regulate each other's effect on epithelial cells presumably to avoid excessive damage to host gastric cells during infection (Argent *et al.*, 2008). Both CagA and VacA have been shown to localise to lipid rafts (Asahi *et al.*, 2003; Nakayama *et al.*, 2006), where CagA associates with tyrosine phosphorylated GIT1/Cat-1 (G protein-coupled receptor kinase-interactor 1/Cool-associated, tyrosine-phosphorylated 1). GIT1 is an important scaffold protein involved in signalling mechanisms that regulate

cytoskeletal dynamics and membrane trafficking (reviewed in Hoefen and Berk, 2006), processes central to hummingbird formation and vacuolation. GIT1 is phosphorylated by Src and FAK kinases, the latter inactivated by phosphorylated CagA (Tsutsumi *et al.*, 2006), and is dephosphorylated by receptor-like protein-tyrosine phosphatase 6 (RPTB6), the receptor for VacA. Mutagenesis studies have shown that CagA decreases the tyrosine phosphorylation of GIT1 (Asahi *et al.*, 2003), and VacA binding to RPTB6 (see Section 1.4.3) has been shown to increase the tyrosine phosphorylation of GIT1 (Fujikawa *et al.*, 2003). Thus functional interaction between CagA and VacA may be acting through GIT1-regulated signalling. There may also be interaction between these two virulence factors acting through NFAT signalling. As mentioned earlier, CagA upregulates the expression of NFAT-regulated genes through stimulation of calcineurin (Yokoyama *et al.*, 2005). However, the anion channel properties of VacA interfere with the calcium ion influx required for calcineurin activity, reducing NFAT activation (see Section 1.6.4)(Boncristiano *et al.*, 2003; Gebert *et al.*, 2003). In addition to differential effects on signalling pathways, CagA has also been shown to inhibit the endocytosis of VacA, reducing the toxin's ability to induce vacuolation and apoptosis (Akada *et al.*, 2010; Oldani *et al.*, 2009). If VacA channels in the plasma membrane benefit the bacteria by releasing nutrients or urea, there may be a selective advantage in inhibiting its endocytosis. CagA has also recently been shown to increase the uptake of transferrin across the basolateral surface of polarised epithelial cells, while VacA redirects the internalised transferrin/transferrin receptor complex to the apical surface of the cell (Tan *et al.*, 2011). In this way, CagA and VacA may work together to modify iron trafficking to support the growth of *H. pylori* microcolonies on the epithelial surface.

1.3.2 Duodenal ulcer promoting gene A (*dupA*)

In a recent study of 500 *H. pylori* strains from East Asia and South America, Lu *et al*. (2005a) identified a novel *H. pylori* gene which was present at a significantly higher frequency among strains isolated from duodenal ulcer patients compared with gastritis only patients. Named the duodenal ulcer promoting gene A (*dupA*), it was also shown that its presence was a marker for increased antral neutrophil infiltration and IL-8 secretion, and was associated with reduced gastric atrophy, intestinal metaplasia and gastric cancer risk (Lu *et al.*, 2005a). *dupA*

represents loci *jhp0917* and *jhp0918* of the strain J99 genome sequence, and is located within a region of high gene variability and different G+C content suggestive of acquired DNA. Further studies have also shown an association between *dupA* and duodenal ulceration in Iraq (Hussein *et al.*, 2008) and North India (Arachchi *et al.*, 2007). However, no association with *H. pylori*-related disease was found among patients from Iran (Douraghi *et al.*, 2008) or Brazil (Gomes *et al.*, 2008), although *dupA* prevalence was very high in the latter population. Interestingly, *dupA* negative strains were associated with premalignant lesions such as dysplasia and lymphoid follicle formation in the Iranian study (Douraghi *et al.*, 2008) in agreement with Lu *et al*.'s (2005a) finding that *dupA* is associated with reduced gastric cancer risk. Conversely, Argent *et al*. (2007) found that *dupA* was associated with gastric cancer, but not duodenal ulceration in a collection of strains from Belgium, South Africa, China and North America. To overcome the limitations of small sample size, two recent meta-analysis studies found that *dupA* prevalence, and its association with disease differed geographically, and worldwide, an association between *dupA* positivity and duodenal ulcer was confirmed, but no overall association with gastric ulcer or gastric cancer was found (Hussein, 2010; Shiota *et al.*, 2010).

1.3.3 Outer inflammatory protein A (OipA)

Mutagenesis of genes annotated as putative outer membrane proteins in the *H. pylori* genome sequence identified a novel gene encoding a 34 kDa protein involved in stimulating AGS cells to secrete IL8, and hence named outer inflammatory protein A (OipA, also known as HopH)(Yamaoka *et al.*, 2000a). However, other mutagenesis studies have failed to find an involvement of OipA in *H. pylori*-induced epithelial IL-8 secretion (Akanuma *et al.*, 2002; Ando *et al.*, 2002a; Dossumbekova *et al.*, 2006; Odenbreit *et al.*, 2002b). Some strains have a non-functional version of *oipA* containing frameshift mutations within a dinucleotide repeat region indicative of phase variation by a slipped-strand repair mechanism (Yamaoka *et al.*, 2000a). While *oipA* 'on' strains are more frequently isolated from patients with more severe gastric disease, such strains are commonly *cagA* positive and have the more pathogenic allelic forms of other virulence factors such as *vacA* and *babA*, therefore it is difficult to evaluate the importance of *oipA* status in determining *H. pylori* disease risk (Ando *et al.*, 2002b;

Dossumbekova *et al.*, 2006). However, one multivariate analysis of *H. pylori* clinical isolates from North and South America showed that *oipA* 'on' status was significantly associated with duodenal ulceration, high *H. pylori* density and increased gastric inflammation independently from other virulence factors (Yamaoka *et al.*, 2002a). Furthermore, inactivation of *oipA* has been shown to reduce the development of dysplasia and gastric adenocarcinoma in a gerbil model of *H. pylori* infection (Franco *et al.*, 2008). Mutagenesis studies suggest that OipA may function as an adhesin (Dossumbekova *et al.*, 2006), although its role in colonisation of animal models is unclear (Akanuma *et al.*, 2002; de Jonge *et al.*, 2004a; Franco *et al.*, 2008; Yamaoka *et al.*, 2002b). A recent study has shown that a *H. pylori oipA* null mutant strain was unable to induce full phosphorylation and activation of FAK in gastric epithelial cells, preventing *H. pylori*-induced actin stress fibre formation and changes in cell morphology (Tabassam *et al.*, 2008). Interestingly, the effects of *oipA* mutagenesis on FAK phosphorylation were greater than those observed for a *cag* PAI deletion mutant. OipA has also been shown to affect the nuclear translocation of beta-catenin in human gastric cells co-cultured with *H. pylori* (Franco *et al.*, 2008), and is associated with increased levels of the proinflammatory cytokines IL-18 (Yamauchi *et al.*, 2008), IL-6 (Lu *et al.*, 2005b) and RANTES (Regulation on Activation, Normal T-cell Expressed and Secreted)(Kudo *et al.*, 2005) both *in vitro* and *in vivo*.

1.3.4 Adhesins

Adhesion to host cells is an important aspect of bacterial colonisation of the gastrointestinal tract as it helps to prevent expulsion due to the movement of material through the gut, and allows close contact for the efficient delivery and action of other virulence factors, such as toxins. While the majority of *H. pylori* bacteria reside within the mucus layer overlying the epithelium, a proportion are found attached to epithelial cells, and such adhesion has been associated with increased histological disease activity (Hessey *et al.*, 1990). Two of the most studied *H. pylori* adhesins are the outer membrane proteins BabA and SabA. BabA allows *H. pylori* to bind strongly to fucosylated Lewis b (Le^b) blood group antigen structures which are found on the surface of epithelial cells, as well as on mucin MUC5AC, one of the major glycoproteins of the mucus layer overlying the gastric epithelium (Ilver *et al.*, 1998; Linden *et*

al., 2002). It has been shown that *H. pylori* co-localises with MUC5AC in gastric biopsies from *H. pylori*-infected patients (Van den Brink *et al.*, 2000). The gene *babA* exists in two main allelic forms: *babA1*, which lacks the translational start site and signal peptide, and *babA2*, which encodes the full length, active protein (Backstrom *et al.*, 2004) capable of binding Le^b. Expression of functional BabA protein can be modulated by recombination between *babA* alleles and a closely related locus *babB* (Backstrom *et al.*, 2004; Solnick *et al.*, 2004). The presence of the *babA2* allele has been associated with increased *H. pylori* colonisation density, higher inflammation and more severe gastric disease, particularly in combination with other virulence determinants such as *cagA* and the *vacA* s1 allele (Gerhard *et al.*, 1999; Prinz *et al.*, 2001; Rad *et al.*, 2002; Thoreson *et al.*, 2000; Yamaoka *et al.*, 2002c; Yu *et al.*, 2002). The increased adherence of *babA2*-type *H. pylori* may enhance the virulent actions of CagA and VacA s1-type toxin.

The adhesin SabA was identified through the study of the adherence properties of a *babA1A2* null mutant strain (Mahdavi *et al.*, 2002). SabA binds to sialyated Lewis x (sLe^x) antigen on host membrane glycolipids which is present at a higher level in the inflamed gastric mucosa, and can also bind sialyated Lewis a antigen (Mahdavi *et al.*, 2002). Sialyated glycans are more abundant in transformed tissue, and high expression of dimeric sLe^x in gastric adenocarcinoma is associated with metastasis and poor prognosis (Amado *et al.*, 1998). SabA may allow *H. pylori* to colonise inflamed or dysplastic mucosal sites. Interestingly, *H. pylori* can alter the expression of host glycan biosynthesis genes leading to increased levels of sLe^x, and consequently its own adherence (Marcos *et al.*, 2008). SabA is able to bind to sialyated receptors on neutrophils inducing phagocytosis and the release of reactive oxygen species (Petersson *et al.*, 2006; Unemo *et al.*, 2005). It has also been shown that SabA has haemaglutination properties by allowing binding of *H. pylori* to sialyated erythrocytes in gastric mucosal blood capillaries of infected patients (Aspholm *et al.*, 2006). Like *oipA*, the expression of functional SabA adhesin is subject to phase variation owing to the presence of dinucleotide repeats near the start of the gene (Mahdavi *et al.*, 2002). SabA 'ON' status has been associated with severe neutrophil infiltration and atrophy in Japan (Yanai *et al.*, 2007), while expression of SabA protein was less common among strains isolated from duodenal ulcer patients from South America and USA compared with strains

from gastritis or gastric cancer patients (Yamaoka *et al.*, 2006). Duodenal ulceration is associated with higher gastric acid secretion, and interestingly reduced SabA expression has been observed under mildly acidic culturing conditions (Yamaoka *et al.*, 2006). A *sabA* homologue has also been identified in the *H. pylori* genome, and named *sabB* (Mahdavi *et al.*, 2002). *sabB* expression is also subject to phase variation, and 'OFF' status has been associated with duodenal ulceration (de Jonge *et al.*, 2004b).

Two further adhesins encoded by adjacent genes *alpA* and *alpB* have been shown to be involved in binding to human gastric tissue, and are required for colonisation of animal models (de Jonge *et al.*, 2004a; Lu *et al.*, 2007; Odenbreit *et al.*, 2002a). AlpAB adhesins from East Asian and Western *H. pylori* strains have been found to differ in their ability to modulate proinflammatory signalling cascades, which may relate to geographical differences in disease outcome (Lu *et al.*, 2007).

1.3.5 Other colonisation factors

One of the most important aspects of *H. pylori* colonisation of the human stomach is its ability to resist the acidic conditions of the gastric lumen. *H. pylori* is not an acidophile, and normally colonises deep within the gastric mucosa where the pH is close to neutral. However, sporadic episodes of acid shock are likely, such as during initial colonisation and upon damage to the mucus layer. *H. pylori* can tolerate acidic exposures as low as pH 1 for several hours (Stingl *et al.*, 2001), and can grow at pH 5.5 to 8.5 (Morgan *et al.*, 1987). The hydrolysis of urea to carbon dioxide and ammonia by urease is essential for *H. pylori* acid tolerance *in vitro* (Marshall *et al.*, 1990), and for colonisation of animal models (Eaton *et al.*, 1991; Tsuda *et al.*, 1994). A high level of urease activity is characteristic of *H. pylori*, and this feature is often used for its identification both in the laboratory and clinically. The urease enzyme is composed of two subunits, UreA and UreB, and is primarily a cytoplasmic protein, although surface-bound urease has been reported, presumably adsorbed following its release from lysed bacteria (Bode *et al.*, 1993; Hawtin *et al.*, 1990). A third protein, UreI, forms a proton-gated urea channel regulating intracellular urea concentrations (Weeks *et al.*, 2000). It was originally suggested that surface-bound urease protected *H. pylori* against the low pH of the stomach by neutralizing acid within the immediate surroundings of the bacterium (Dunn *et al.*,

1997). However, it is now thought to be more likely that protection derives from internal urease activity maintaining either cytoplasmic (Stingl *et al.*, 2001) or periplasmic pH (Scott *et al.*, 1998).

Another important enzyme group which regulate pH homeostasis are carbonic anhydrases which catalyse the reversible hydration of carbon dioxide to bicarbonate. The *H. pylori* genome encodes two carbonic anhydrases belonging to the a (HP1186) and b (HP0004) families (Tomb *et al.*, 1997). The *H. pylori* a-carbonic anhydrase (a-CA) is located within the periplasm, and is essential for *H. pylori* survival under acidic conditions (Marcus *et al.*, 2005). It is proposed that the enzyme complements cytoplasmic urease activity by converting carbon dioxide diffusing into the periplasm to bicarbonate, which allows buffering of the periplasm at pH 6.1. The concerted action of urease and a-CA in acid survival is further suggested by the acid-induced expression of both enzymes, regulated by the two component acid-responsive signalling system, ArsRS (Pflock *et al.*, 2005; Wen *et al.*, 2007). The ArsRS two component system also regulates other enzymes involved in ammonia production including the amidase AmiE and the formamidase AmiF, as well as the urea-producing arginase RocF (Pflock *et al.*, 2006). While these enzymes may contribute to acid tolerance, they are not essential for colonisation of a mouse model (Bury-Mone *et al.*, 2003; McGee *et al.*, 1999).

H. pylori strains possess multiple polar flagella which are necessary for colonisation of the viscous mucus layer overlying the gastric epithelium. The flagella are composed of two subunits FlaA and FlaB, and many other proteins are involved in the regulation, secretion and operation of the flagella apparatus. *H. pylori* follows a pH gradient within the mucus layer from the more acidic lumen side to the more neutral epithelium, and motility along this gradient is essential to avoid clearance due to the rapid turnover of mucus (reviewed in Clyne *et al.*, 2007). A chemotactic response to urea and bicarbonate has also been demonstrated (Mizote *et al.*, 1997). Motility, pH taxis and chemotaxis are essential functions for persistent gastric colonisation of animal models (Croxen *et al.*, 2006; Eaton *et al.*, 1992; Foynes *et al.*, 2000; McGee *et al.*, 2005).

1.3.6 Lipopolysaccharide

The biological activity of *H. pylori* lipopolysaccharide (LPS) on the host innate immune system is poor compared with enteric Gram-negative bacteria (reviewed in Moran, 2007; Muotiala *et al.*, 1992), and it is generally viewed that it does not make a major contribution to *H. pylori*-induced inflammation. Differences in the lipid A moiety of *H. pylori* LPS compared with other bacterial LPS structures may account for this lower immunoactivity (Moran *et al.*, 1997). An interesting feature of *H. pylori* LPS is the presence of Lewis x and y antigens on the O-antigen polysaccharide side chain (Aspinall and Monteiro, 1996; Aspinall *et al.*, 1996). Lewis x and y blood group antigens are expressed in the normal gastric mucosa (Kobayashi *et al.*, 1993; Sakamoto *et al.*, 1989), and it has been suggested that mimicry between LPS and host Le antigens is one mechanism which *H. pylori* uses to evade the host immune system, contributing to the persistence of infection (Aspinall and Monteiro, 1996; Aspinall *et al.*, 1996). Alternatively, *H. pylori* Le antigens may promote an autoimmune response directed towards the gastric mucosa which could play a role in the pathogenesis of infection (Appelmelk *et al.*, 1996; Aspinall *et al.*, 1996).

1.4 The vacuolating cytotoxin, VacA

The presence of a cytotoxic activity in *H. pylori* broth culture supernatants was first reported two decades ago (Leunk *et al.*, 1988). The authors observed that broth culture supernatants from approximately half of the clinical isolates studied induced extensive cytoplasmic vacuolation of a variety of epithelial cell lines (see Figure 1). The clinical importance of this vacuolating activity was soon realised when it was shown that strains producing this effect *in vitro* were more commonly isolated from patients with peptic ulcer disease (Figura *et al.*, 1989; Tee *et al.*, 1995). Furthermore, *H. pylori*-infected patients were shown to produce a neutralising antibody response to the toxin confirming its expression *in vivo* (Cover *et al.*, 1992a; Pereira Lage *et al.*, 1993). The toxin responsible for this vacuolating activity was successfully purified from *H. pylori* culture supernatant and named the vacuolating cytotoxin, VacA (Cover and Blaser, 1992). In mice, oral administration of *H. pylori* sonicate of a toxigenic strain induced erosive gastric lesions and mononuclear inflammatory cell infiltration

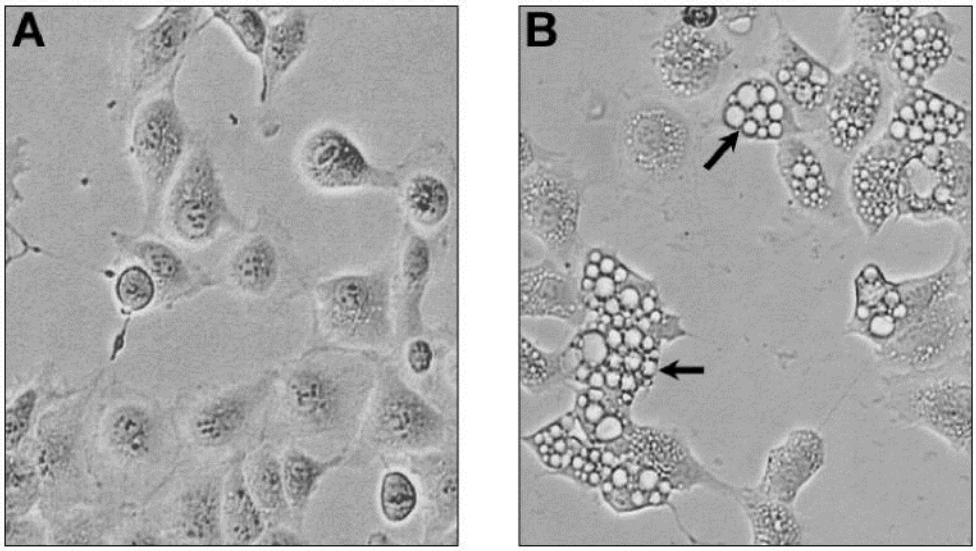


Figure 1 The effect of *H. pylori* culture supernatant on epithelial cells. RK13 rabbit kidney epithelial cells were incubated overnight with either medium alone (A) or a 5 fold dilution of *H. pylori* strain 60190 culture supernatant (B). Black arrows highlight the presence of extensive cytoplasmic v acuolation.

in the lamina propria, while nontoxigenic strain sonicate did not have this effect (Telford *et al.*, 1994). Similarly, oral administration of purified VacA induced the same lesions in the murine gastric mucosa, although extensive inflammation was not observed (Telford *et al.*, 1994). Vacuolisation of the epithelium has also been observed *in vivo* following oral administration of purified toxin to mice (Supajatura *et al.*, 2002). Amino terminal sequence information derived from purified VacA led to the cloning and nucleotide sequence determination of the gene, *vacA*, encoding the toxin from four different toxigenic strains (Cover *et al.*, 1994; Phadnis *et al.*, 1994; Schmitt and Haas, 1994; Telford *et al.*, 1994). Furthermore, these studies showed by hybridisation and PCR experiments that *vacA* is also present in the majority of nonvacuolating strains, although extensive sequence diversity exists between toxigenic and nontoxigenic strains. Since its initial identification, the vacuolating cytotoxin has been the subject of extensive study, addressing aspects such as VacA secretion and delivery, structure, its functional role in *H. pylori* pathogenesis, the mechanisms underlying vacuolating activity, and natural *vacA* polymorphism and its clinical importance.

1.4.1 Mechanisms of VacA secretion and delivery

Evidence that VacA was secreted by an autotransport mechanism initially came from the analysis of its deduced polypeptide sequence. While *vacA* nucleotide sequences vary between strains, the gene is usually about 3.9 kb in size and encodes a polypeptide of approximately 139 kDa (Cover *et al.*, 1994; Phadnis *et al.*, 1994; Schmitt and Haas, 1994; Telford *et al.*, 1994)(see Figure 2). Under denaturing conditions, secreted VacA migrates through polyacrylamide gels with an apparent molecular mass of 87-95 kDa. The molecular mass of purified VacA from strain 60190 has been precisely determined as 88.2 kDa by matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF) (Nguyen *et al.*, 2001). The discrepancy in these sizes is due to *vacA* encoding a precursor polypeptide structure which is typical of an autotransporter mechanism of secretion. In addition to the 87-95 kDa mature, secreted toxin, the precursor polypeptide includes a characteristic N-terminal signal sequence of 33 amino acids and a 33 kDa C-terminal domain which are both proteolytically cleaved during secretion. The secreted passenger domain has been shown to consist of the mature toxin and a 12 kDa domain which is cleaved off following secretion (Bumann *et al.*, 2002; Nguyen *et al.*, 2001). This 12 kDa fragment has been detected in broth culture supernatants of *H. pylori* strain 26695 (Bumann *et al.*, 2002), and is most likely equivalent to the alpha-protein of the prototypical *Neisseria gonorrhoeae* IgA1 protease (Pohlner *et al.*, 1987). The IgA1 protease alpha-protein can enter cells *in vitro* via an endocytic route, and translocate to the nucleus (Pohlner *et al.*, 1995). The alpha protein of *Neisseria meningitides* IgA protease has been shown to be immunogenic, stimulating a helper T-cell response in human peripheral blood monocytes (Jose *et al.*, 2000). It is not known whether the VacA 12 kDa fragment has any pathological role during *H. pylori* infection. The C-terminal domain of the VacA precursor is predicted to contain an amphipathic 3-strand-rich region and terminates in an alternating hydrophobic amino acid motif ending in phenylalanine, which is commonly found in other autotransporters and bacterial outer membrane proteins (Cover *et al.*, 1994). This domain shares 43% similarity with the Iga3-core of the IgA protease of *Neisseria gonorrhoeae* (Schmitt and Haas, 1994), and weak homology with 17 other functionally characterized autotransporters, including the presence of 14 putative amphipathic 13-strands which are typical of outer membrane-spanning domains (Loveless and Saier, 1997).

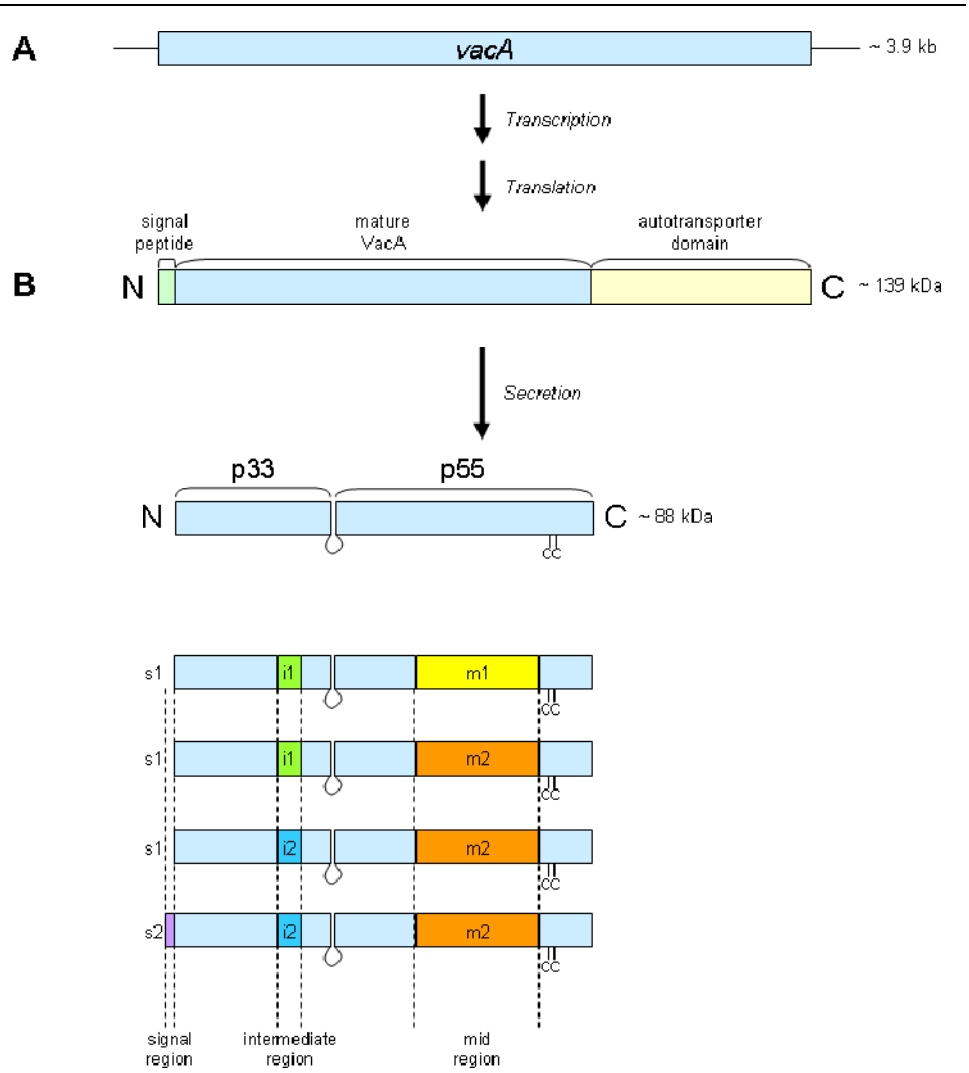


Figure 2 *vacA* encodes an autotransporter. (A) The *vacA* gene is approximately 3.9 kb (varies between strains), and encodes a precursor poly peptide of about 139 kDa. (B) The precursor polypeptide contains a typical signal peptide which directs secretion across the cytoplasmic membrane, and a C-terminal autotransporter domain which directs secretion across the outer membrane. (C) Following N- and C-terminal processing during secretion, the mature secreted VacA protein folds into two domains (p33 and p55) which are linked by an exposed loop. Cleavage may occur at this exposed loop, although the two subunits remain in association. (D) Allelic variation has been described encoding three regions of greater sequence div ersity: the signal, intermediate and mid-regions. Mosaicism exists giving rise to the f our common allelic types shown. s2/m1 variants are rarely observed in populations. A conserved pair of cysteine residues are also shown.

Transposon insertions in the region encoding the 50 kDa C-terminal domain abolished toxin production, supporting its role in toxin autotransport (Schmitt and Haas, 1994). Furthermore, antisera against the C-terminal region of VacA recognised a 33 kDa cell-associated polypeptide by immunoblotting (Telford *et al.*, 1994), and localised to the outer membrane in

immunogold labelling of electron microscopy sections of *H. pylori* (Fiocca *et al.*, 1999), consistent with an autotransport mechanism. The role of the VacA C-terminal cell-associated domain in autotransport function was elegantly confirmed by Fischer *et al.* (2001) by fusing this region to the cholera toxin B subunit, and showing secretion of the latter to the surface of *H. pylori*. Finally, like most autotransporters, VacA is low in cysteine residues, containing a single pair spaced 11 residues apart, and located towards the C-terminus of the secreted toxin. The passenger domains of many autotransporters also contain a pair of cysteine residues, usually with the same spacing and location. These cysteine residues have been shown to be required for high VacA production (Letley *et al.*, 2006 [see Chapter 5]).

In addition to free, secreted VacA, a significant proportion of the toxin is found in association with the bacterial membrane (Telford *et al.*, 1994). Studies have estimated the proportion of the bacterial-associated VacA to be from 20% (Fitchen *et al.*, 2003) to more than 50% (Ilver *et al.*, 2004) of total toxin production. This form of the toxin is of the same molecular mass as the processed, secreted monomeric VacA observed in culture supernatant, and is presumed to occur post-secretion as both *vacA* null mutant bacteria and *E. coli* are able to adsorb VacA from culture supernatant derived from a VacA producing strain (Fitchen *et al.*, 2005; Pelicic *et al.*, 1999). Adsorbed VacA is tightly bound to the surface in discrete foci (Fitchen *et al.*, 2003; Ilver *et al.*, 2004), and this interaction does not seem to be protein or LPS mediated (Fitchen *et al.*, 2005). VacA allelic diversity (see below) does not appear to affect toxin adsorption to bacterial surfaces (Fitchen *et al.*, 2005). The significance of this bacterial-associated VacA is unclear. The adherence of *H. pylori* to polarised monolayers of Madin-Darby canine kidney (MDCK) cells results in a decrease in trans-epithelial resistance across the monolayer, and this effect appears to be mediated by surface-bound VacA (Pelicic *et al.*, 1999). In one study, the adherence of *H. pylori* to AGS cells resulted in vacuolation, but the possibility that this was due to the secretion of VacA by adhered bacteria during the assay was not completely excluded (Ilver *et al.*, 2004). However, contact-dependent direct transfer of adsorbed VacA from bacteria to host cells was observed by immunofluorescence confocal microscopy. In contrast, another study has reported that VacA preadsorbed to *vacA* null mutant bacteria was unable to induce vacuolation of epithelial cells, despite sufficient quantities of toxin being present (Fitchen *et al.*, 2005).

Electron microscopy studies of *H. pylori* broth cultures have identified outer membrane blebbing and the release of outer membrane vesicles (OMVs) of between 50-300 nm in diameter (Fiocca *et al.*, 1999; Keenan *et al.*, 2000; Ricci *et al.*, 2005; Sommi *et al.*, 1998). As OMVs have also been identified in *H. pylori*-infected gastric biopsies, scattered around bacteria and adhering to the epithelium, these structures may represent a potential delivery system for *H. pylori* virulence factors (Fiocca *et al.*, 1999; Keenan *et al.*, 2000). Indeed, OMVs have been shown to contain mature VacA protein bound to their surface, both *in vitro* and *in vivo*, although other virulence factors such as CagA and urease do not seem to be present (Fiocca *et al.*, 1999; Keenan *et al.*, 2000; Ricci *et al.*, 2005; Sommi *et al.*, 1998). OMVs containing bound VacA have been shown to interact closely with the surface of MKN28 cells and to accumulate in endosomes (Fiocca *et al.*, 1999; Ricci *et al.*, 2005; Sommi *et al.*, 1998), and vacuolation of cultured AGS cells by OMV-bound VacA has been reported (Ismail *et al.*, 2003). However, while OMV-bound VacA has been estimated to account for about 25% of the toxin present in broth culture supernatants (Ricci *et al.*, 2005), virtually all the *in vitro* vacuolating activity measured in this study was accounted for by free VacA. Other effects attributed to *H. pylori* OMVs include dose-dependent modulation of cell proliferation, increased IL-8 production and the induction of apoptosis (Ayala *et al.*, 2006; Ismail *et al.*, 2003), although whether delivery of OMVs at concentrations required for some of these effects occurs *in vivo* is unclear.

1.4.2 The oligomeric structure of VacA

VacA is extremely well suited to is gastric environment. Upon exposure to acidic pH, the toxin undergoes structural changes characterised by shifts in its far-UV circular dichroism spectra consistent with a turn to 6-structure transition (de Bernard *et al.*, 1995). These effects are not reversed upon neutralisation, and instead the toxin undergoes further structural changes affecting the pronase and proteinase K digestion profiles of the toxin. More significantly, the structural changes induced by acid exposure strongly enhance the vacuolating activity of the toxin, and this acid activation occurs rapidly and at pH 5.5. Furthermore, at pH 2.0, VacA is highly resistant to the major gastric proteinase, pepsin.

Under denaturing conditions the toxin migrates through polyacrylamide gels with an apparent molecular mass of 87-95 kDa, but in its native form exists as larger, oligomeric structures of about 700-972 kDa (Cover and Blaser, 1992; Manetti *et al.*, 1995). Quick-freeze, deep etch transmission electron microscopy revealed these high molecular mass VacA oligomers to be flower-like structures of ~30 nm diameter, consisting of a central ring of ~1215 nm diameter surrounded by 6 or 7 globular "petals" of ~6 nm (Cover *et al.*, 1997; Lupetti *et al.*, 1996)(see Figure 3A). The proportion of oligomeric structures with 6 or 7 fold radial symmetry is determined by the size of an exposed loop where proteolytic processing into p33 and p55 domains occurs (see Figure 2C)(Burroni *et al.*, 1998)(also refered to as the p37 and p58 domains in the literature). A proportion of the oligomeric ring structures appear flatter than normally observed, with counterclockwise chirality and it was originally suggested that these forms represent proteolytically processed VacA which has lost the p33 subunit (Lanzavecchia *et al.*, 1998; Lupetti *et al.*, 1996). However, an alternative proposal was that the flat ring structures are an artifact of the freeze-fracturing process during sample preparation, and that the normal form of VacA is a dodecameric or heptadecameric structure consisting of two ring structures assembled face-to-face (Cover *et al.*, 1997). Indeed, oligomeric VacA absorbed to mica was observed by electron microscopy to dissociate into 12 or 14 monomers under acidic conditions (Cover *et al.*, 1997). Further support for a bilayer VacA oligomeric structure came from cryo-negative staining electron microscopy studies of purified, soluble VacA oligomers immobilized by vitrification, which, in addition to the ring structures observed previously, also identified rectangular, bilayered structures presumed to be side views of the oligomer (Adrian *et al.*, 2002; El-Bez *et al.*, 2005). Given that the vacuolating activity of the toxin is enhanced by low pH treatment, the disassembly of the oligomeric structure upon exposure to acid may be important for toxin activation (Cover *et al.*, 1997; Molinari *et al.*, 1998a). Following reneutralisation, dissociated VacA monomers are able to reassemble into flower-like oligomeric VacA structures, although they are slightly less ordered. While less significant *in vivo*, oligomeric VacA similarly dissociates upon exposure to alkaline pH, with subsequent reassembly following reneutralisation, and this treatment also increases the vacuolating activity of the toxin (Yahiro *et al.*, 1999).

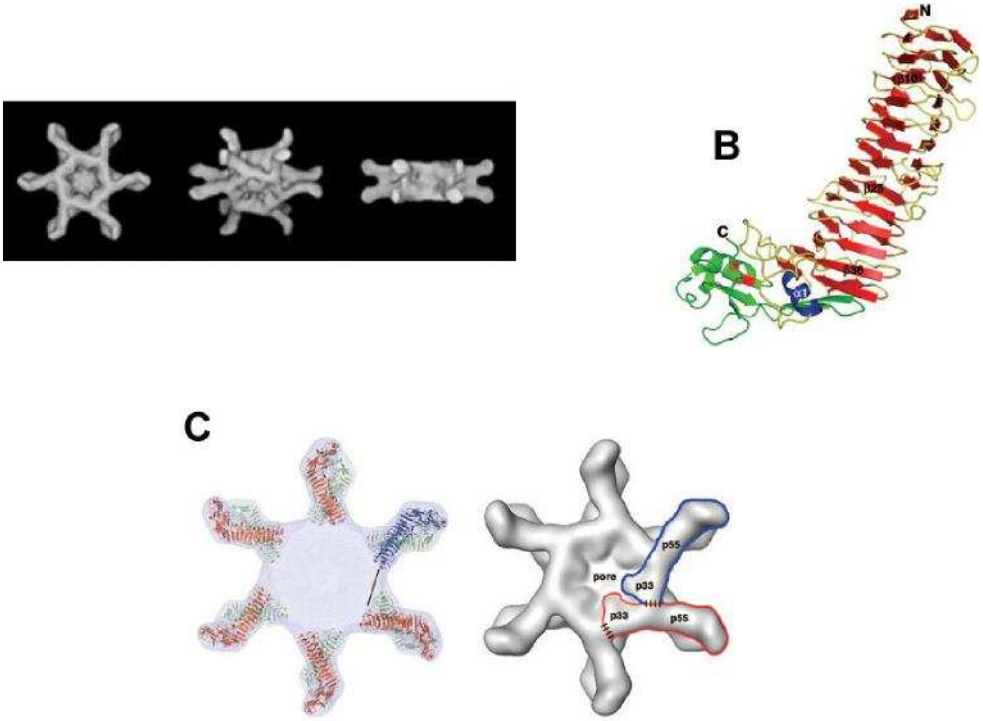


Figure 3 The structure of the vacuolating cytotoxin, VacA. (A) 3D reconstruction of dodecameric VacA deduced from cryo-negative staining electron microscopy data (taken from El-Bez *et al*., 2005). The top view (*left*) shows the 6-f old symmetry, and the side view (*right*) shows the bilay ered structure. (B) Crystal structure of the purified p55 VacA domain (taken from Gangwer *et al*., 2007). The structure is predominately a right-handed parallel 13-helix with a C-terminal domain consisting of both ü and 13 structure, with a single disulphide bond. (C) The p55 crystal structure fits in to the 'petals' of the flower-like oligomeric structure (*left*). The black line indicates the predicted space occupied by the p33 domain if it extends the p55 13-helix. The proposed position of VacA monomers in the oligomeric structure (*right*). Inter-molecular interaction between the p33 domain of one monomer and the p55 domain of its neighbour are shown by dashed lines (taken from Gangwer *et al*., 2007).

The location of the p33 and p55 domains within the oligomeric structure of the toxin may be deduced from structural studies of the purified p55 domain. Expression of the p55 subunit alone in *H. pylori* results in the secretion of soluble p55 dimers which bind to target cells but are not internalized and do not induce vacuolating activity (Reyrat *et al.*, 1999). Comparing 3D images of these p55 dimers with that of the wild type oligomer, reconstructed from deep-etch electron microscopy images, suggests that the peripheral arms of the oligomer are mainly composed of p55 domains, and therefore the central ring is made up from p33 domains (Reyrat *et al.*, 1999). More recently, the crystal structure of the p55 domain

has been resolved to 2.4 ~, revealing a predominantly right-handed parallel 13-helix structure with a C-terminal globular domain containing both and 13 secondary structural elements (Gangwer *et al.*, 2007)(see Figure 3B). Within the crystal, p55 structures were arranged in pairs, consistent with their dimerisation upon purification. Docking of the p55 crystal structure into the electron microscopy-derived image of the dodecamer (El-Bez *et al.*, 2005) agrees with the peripheral arms of the oligomeric structure being composed of the p55 subunit (Gangwer *et al.*, 2007)(see Figure 3C). The current model proposes that VacA monomers are intercalated with each other in the oligomeric form, with interactions occuring between N-terminal residues of the p33 domain of one monomer and the N-terminus of the p55 domain of a neighbouring monomer (Gangwer *et al.*, 2007; Reyrat *et al.*, 1999). As proteolytically cleaved VacA still elutes in the same high molecular mass fraction as intact VacA by gel filtration chromatography (Torres *et al.*, 2004), noncovalent intramolecular interaction must also occur between p33 and p55. Such interactions have been shown to occur between p33 and p55 recombinant fragments using yeast two hybrid technology, although homomeric interaction was not observed for either domain (Torres *et al.*, 2004). Furthermore, while neither domain is able to interact with intact VacA monomers independently, immunoprecipitation experiments show that a p33/p55 mixture does interact with monomeric VacA, and that p55 is able to complex with two p33 domains simultaneously (Torres *et al.*, 2005). Multiple sites of interaction between p33 and p55 domains is also supported by yeast two hybrid studies using a mutated p33 domain containing a small in-frame deletion which prevents oligomerisation (Genisset *et al.*, 2006). This mutation reduced p33/p55 interactions two fold, consistent with loss of intermolecular, but not intramolecular VacA interactions. The crucial role that p33/p55 interactions play in VacA oligomer assembly is further suggested by the observation that VacA in-frame deletions which prevent the formation of oligomers also disrupt p33/p55 interactions in a yeast two hybrid system (Torres *et al.*, 2004).

VacA has been shown bind to liposomes (Molinari *et al.*, 1998a), the plasma membrane of HeLa cells (Garner and Cover, 1996; Torres *et al.*, 2005), and recombinant VacA subunits interact with unilamellar liposomes (Moll *et al.*, 1995). Membrane insertion of VacA is strongly increased under mild acidic pH conditions, probably due to the exposure of hydrophobic sites (Molinari *et al.*, 1998a; Moll *et al.*, 1995). Given its interaction with

membranes, the structure of the toxin in association with lipid bilayers and cell membranes has also been studied. Atomic force microscopy of purified VacA bound to pure, supported lipid bilayers revealed hexameric ring structures of similar dimension to those observed by electron microscopy of VacA bound to mica (Czajkowsky *et al.*, 1999; Geisse *et al.*, 2004). Similarly, deep-etch electron microscopy of VacA bound to rabbit erythrocytes showed the same flower-shaped structures as the flat, counterclockwise forms observed previously (Adrian *et al.*, 2002). The formation of these oligomeric structures on membrane surfaces also required either mild acidic conditions (Czajkowsky *et al.*, 1999; Geisse *et al.*, 2004) or acid-activated toxin (Adrian *et al.*, 2002).

The study of mutated VacA variants has provided further insights into the role of toxin oligomerisation. VacA(A6-27), a mutated form of the toxin which is unable to induce vacuolation, is still able to assemble into oligomeric complexes within lipid bilayers (Vinion-Dubiel *et al.*, 1999), but structural alterations exist in the central ring area, implicating this part of the structure in toxin activity (El-Bez *et al.*, 2005). The region deleted in VacA(A6-27) includes three tandem G*XXX*G motifs, characteristic of transmembrane helix-helix associations (Russ and Engelman, 2000), and shown to mediate transmembrane protein dimerization using the recombinant TOXCAT system in *E. coli* (McClain *et al.*, 2001a). Alanine substitutions within these motifs showed that the first G*XXX*G motif was essential for VacA activity (McClain *et al.*, 2003), and a model has been proposed in which the G*XXX*G motifs from adjacent monomers within the hexameric toxin structure allow helix-helix packing to form a transmembrane channel (Kim *et al.*, 2004). Interestingly, VacA(A6-27) has a dominant negative effect on wild type VacA, which probably results from the formation of mixed oligomeric complexes (Vinion-Dubiel *et al.*, 1999). Another dominant negative mutant, VacA(A49-57), is unable to oligomerise itself and prevents the reassembly of wild type VacA oligomers following acid activation (Genisset *et al.*, 2006). The dominant negative effects that these mutants have on the vacuolating activity of wild type VacA clearly emphasise the important role of VacA oligomerisation in toxin activity.

1.4.3 VacA binding to epithelial cells and its internalisation

Previous studies of VacA binding to target epithelial cells have given conflicting results. Massari et al (1998) demonstrated saturable binding of VacA to HeLa cells using flow cytometry, consistent with a high affinity receptor for the toxin on the cell surface. In contrast, binding studies using ^{125}I-labelled VacA showed that a large proportion of toxin binding to HeLa cells was nonspecific and saturable binding indicative of a single high affinity receptor was not observed (McClain *et al.*, 2000; Ricci *et al.*, 2000). Similarly, acid activation of VacA was observed to increase the binding of the toxin to AZ-521 cells (Yahiro *et al.*, 1999), a human gastric adenocarcinoma cell line, but not to HeLa cells (Massari *et al.*, 1998; McClain *et al.*, 2000). Immunoprecipitation experiments identified a protein of 140 kDa which coprecipitated with VacA in cell lysates from the VacA-sensitive cell lines AZ-521, AGS (both human gastric adenocarcinoma cell lines) and COS-7 (a monkey kidney cell line), but not HL-60 cells, a human leukemic cell line which is not sensitive to VacA-induced vacuolation (Yahiro *et al.*, 1997). Proteins of molecular mass 250 kDa and 150 kDa also coprecipitated with VacA in AZ-521 and COS-7 cells, respectively, suggesting the presence of more than one VacA receptor. Following purification of the 140 and 250 kDa VacA-binding proteins, peptide sequence analysis led to their identification as the glycoproteins receptor-like protein-tyrosine phosphatase a (RPTPa) (Yahiro *et al.*, 2003) and RPTPl3 (Yahiro *et al.*, 1999), respectively.

There are several lines of evidence supporting a role for RPTPl3 as a VacA receptor. Differentiation of HL-60 cells into macrophage-like cells by treatment with phorbol 12-myristate 13-acetate (PMA) induces VacA sensitivity (de Bernard *et al.*, 1998b), and this is accompanied by greatly increased amounts of RPTPl3 mRNA and the appearance of RPTPl3 protein (Padilla *et al.*, 2000). The same authors also showed that blocking RPTPl3 expression using antisense oligonucleotides inhibits PMA-induced vacuolation, that RPTPl3 and VacA colocalise on the surface of PMA-treated HL-60 cells, and transfection of BHK-21 cells with RPTPl3 confers strong VacA sensitivity on this cell line. VacA binding to RPTPl3 involves a glycosylated region within the extracellular domain of the receptor (Yahiro *et al.*, 2004). RPTPl3 is mainly located in non-lipid raft microdomains of the plasma membrane of AZ-521 cells, but translocates to lipid rafts following VacA binding, a characteristic which is essential

for subsequent VacA internalisation (Nakayama *et al.*, 2006). RPTPI3 is expressed in the human and the mouse gastric mucosa, and VacA has been shown to bind to this receptor in mouse gastric biopsies (Fujikawa *et al.*, 2003). Mice deficient in RPTPI3 expression do not develop gastric ulcers following oral administration of purified VacA, unlike wild type, RPTPI3 positive mice. Interestingly, VacA is still internalised in the gastric epithelial cells of these RPTPI3-/- mice and cells cultured from these animals are sensitive to VacA-induced vacuolation, most likely due to the presence of other VacA receptors such as RPTPCL. These results indicate that VacA–induced gastric ulceration is independent of vacuolation, and may occur through RPTPI3-mediated cell signalling. GIT1/Cat-1 is a substrate for RPTPI3 (Kawachi *et al.*, 2001), and VacA, acting as a ligand for the receptor, is able to increase GIT1 tyrosine phosphorylation in a dose-dependent manner in BHK-21 cells transfected with RPTPI3 (Fujikawa *et al.*, 2003). GIT1 is a multidomain protein involved in diverse cellular processes including regulating cytoskeletal dynamics during cell migration, receptor internalisation and membrane trafficking (reviewed by Hoefen and Berk, 2006).

Evidence that RPTPCL acts as a receptor for VacA came from the study of toxin-induced vacuolation of G401 cells, a human kidney tumor cell line. Yahiro et al (2003) observed that this cell line did not express RPTPI3 protein or mRNA, yet was still sensitive to VacA-induced vacuolation. A 140kDa protein observed in coprecipitation experiments with an anti-VacA antibody was identified as RPTPCL by peptide sequence analysis. Treating G401 cells with an RPTPCL antisense oligonucleotide markedly reduced VacA-induced vacuolation and toxin internalization. Furthermore, COS-7 cells transfected with RPTPCL cDNA showed a marked increase in RPTPCL expression, and increased VacA binding. In mice, RPTPCL is most abundant in the brain and kidney, but is found at lower levels in most tissue (Sap *et al.*, 1990). A role for RPTPCL in cell-substratum adhesion has been suggested, by regulating Src family kinase activation (Harder *et al.*, 1998).

In addition to binding to high affinity protein receptors, VacA has been found to bind to the glycosphingolipids, galactosylceramide (GalI31Cer) and galabiosylceramide (GalCL4GalI31Cer), isolated from human gastric tissue (Roche *et al.*, 2007). The p33 domain of VacA appears to be required for glycosphingolipid-binding. Binding to these short chain

glycosphingolipids following initial interaction with a high affinity receptor may provide close membrane association required for toxin internalisation. Recombinant p55 VacA subunit has been shown to bind to the surface of RK-13 cells, but unlike full-size VacA is not subsequently internalised (Reyrat *et al.*, 1999). Weak binding of individual p33 and p55 recombinant subunits to HeLa cells has also been observed, but again no internalisation occurred (Torres *et al.*, 2005). However, when both subunits are added to HeLa cells simultaneously, binding is greatly enhanced and both are internalised. Internalisation of VacA into target cells is a slow and temperature-dependent process (Garner and Cover, 1996). While purified toxin is able to bind to cells, internalisation requires the toxin to be in its acid or alkali-activated state (McClain *et al.*, 2000). A requirement for actin is consistent with toxin internalisation occurring through an endocytosis pathway, but the mechanism is not clathrin-dependent (Gauthier *et al.*, 2005; Ricci *et al.*, 2000). VacA preferentially associates with membrane microdomains enriched for cholesterol, sphingolipids and glycosylphosphatidylinositol-anchored proteins (GPI-APs) refered to as lipid rafts (Geisse *et al.*, 2004; Kuo and Wang, 2003; Ricci *et al.*, 2000; Schraw *et al.*, 2002). This association is enhanced upon acid activation and is required for internalisation and vacuolating activity. The association appears to be cholesterol-dependent, but the requirement for GPI-APs is less clear. Removal of GPI-APs from cell membranes does not affect the localisation of VacA to lipid rafts, but inhibits toxin internalisation, the formation of VacA chloride channels (see below) and vacuolating activity (Gauthier *et al.*, 2004; Kuo and Wang, 2003; Ricci *et al.*, 2000). However, this may be due to other changes in lipid raft properties as a GPI-AP-deficient cell line was still sensitive to VacA internalisation and activity (Schraw *et al.*, 2002). VacA internalisation appears to be an actin-dependent process (Gauthier *et al.*, 2004), and it has been suggested to occur through an endocytic pathway used for recycling GPI-APs (Kuo and Wang, 2003). Indeed, the mechanism of VacA internalisation appears to be different to other known lipid raft-dependent endocytic pathways, and its dependence on Cdc42 (cell division cycle 42), a Rho family GTPase involved in regulating pinocytosis of GPI-APs into GPI-AP-enriched early endosomal compartments (GEECs), suggests that the toxin uses the same route to enter cells (Gauthier *et al.*, 2005; Sabharanjak *et al.*, 2002).

Following initial internalisation into GEECs, VacA must locate to the late endosomal compartment from which vacuoles originate (see below). This occurs by transfer of VacA from GEECs to early endosomes which are associated with polymerised actin structures via a CD2-associated protein (CD2AP) bridge (Gauthier *et al.*, 2007). Early endosomes containing VacA are most likely routed to late endosomes via these dynamic actin structures, as CD2AP, which regulates actin polymerisation, is essential for trafficking of VacA from GEECs to late endosomes. VacA may also be able to enter late endosomes directly from the cytosol since either microinjection of VacA, or its intracellular expression, results in the same vacuolated morphology as observed with externally delivered toxin (de Bernard *et al.*, 1998a). Some VacA may also be further routed to the Golgi apparatus since disruption of this compartment with Brefeldin A enhances the vacuolating activity of the toxin (Argent *et al.*, 2004b), and perinuclear co-localisation of the toxin with the Golgi marker, wheat germ agglutinin, has been observed in CHO (Chinese hamster ovary) cells by confocal microscopy (Kuo and Wang, 2003).

1.5 The mechanism of VacA-induced cytoplasmic vacuolation

The observation that *H. pylori* broth culture supernatants induced extensive cytoplasmic vacuolation of several epithelial cell lines (Leunk *et al.*, 1988) eventually led to the identification of VacA, the secreted factor responsible for this effect (Cover and Blaser, 1992). It was later shown that VacA also induced vacuolation of primary human gastric and jejunal mucosal epithelial cells (Harris *et al.*, 1996; Pagliaccia *et al.*, 1998; Smoot *et al.*, 1996). In addition to the toxin, the formation of VacA-induced vacuoles requires an active vacuolar-type-ATPase (Cover *et al.*, 1993; Papini *et al.*, 1993a; Papini *et al.*, 1993b; Papini *et al.*, 1996), an enzyme responsible for acidifying intracellular compartments, and is potentiated by the presence of weak bases such as ammonium chloride (Cover *et al.*, 1992c). Neutral red dye, a weak base, avidly accumulates in VacA-induced vacuoles indicating that they are acidic compartments (Cover *et al.*, 1992b). The vacuoles contain extracellular fluid-phase material suggesting an endocytic origin (Papini *et al.*, 1994), and there are several lines of evidence that suggest that they are derived from late endosomal and lysosomal compartments. VacA-induced vacuoles do not contain transferrin receptor or cathepsin D suggesting that elements

of the recycling and trans-golgi network trafficking pathways are absent, and their formation is inhibited by nocodazole and colchicine, drugs which prevent early to late endosomal trafficking, all consistent with a prelysosomal origin (Papini *et al.*, 1994). The small GTPases Rac1 and Rab7 are both present on VacA-induced vacuoles, and their activity is essential for vacuole formation (Hotchin *et al.*, 2000; Molinari *et al.*, 1997; Papini *et al.*, 1994; Papini *et al.*, 1997). The functions of Rac1, a member of the Rho family of GTPases, include regulation of the actin cytoskeleton and membrane trafficking. Rab7 is present on late endosomes where it regulates their fusion. Vacuoles also contain the lysosomal marker Lgp110, and lack CI-M6PR, another late endosome marker suggesting they result from heterotypic fusion between endosomal and lysosomal compartments (Molinari *et al.*, 1997). Consistent with a hybrid origin is the observation that VacA induces changes in the protein content of late endosome-enriched cell fractions (Molinari *et al.*, 1997). The redistribution of lysosomal membrane proteins occurs at low VacA concentrations in the absence of visible vacuolation, and thus the effect of VacA on the endocytic pathway most likely represents an early event during intoxication, rather than being an artifact resulting from the presence of large vacuoles forcing changes in cell structure (Molinari *et al.*, 1997).

VacA has been shown to form hexameric, gated channels in both planar phospholipid bilayers and the plasma membrane of HeLa cells (Czajkowsky *et al.*, 1999; Czajkowsky *et al.*, 2005; Iwamoto *et al.*, 1999; Szabò *et al.*, 1999; Tombola *et al.*, 1999a; Tombola *et al.*, 1999b). VacA channels exhibit a low conductance with moderate anion selectivity, maintained from pH 4 to 12, and their formation requires acidic conditions or activated toxin, suggesting assembly from monomers rather than direct insertion of oligomeric VacA. The electrophysiological properties of the VacA channel resemble those of host chloride channels (Czajkowsky *et al.*, 2005; Iwamoto *et al.*, 1999), and channel activity can be blocked by a variety of chloride channel inhibitors including 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) (Iwamoto *et al.*, 1999; Szabò *et al.*, 1999; Tombola *et al.*, 1999b; Tombola *et al.*, 2000). These inhibitors also prevent, and partially reverse, VacA-induced vacuolation, implying that channel formation is essential for vacuolating activity. The current model for VacA induction of vacuolation (see Figure 4)(Iwamoto *et al.*, 1999; Szabò *et al.*, 1999; Tombola *et al.*, 1999a) proposes that

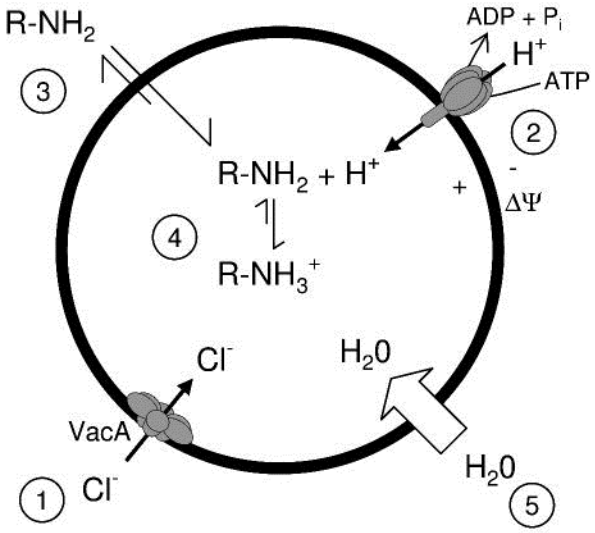


Figure 4 The proposed model of VacA-induced cytoplasmic vacuolation. VacA channels are internalised into late endosomal compartments leading to the influx of chloride ions (1). Subsequent reduction of the inside-positive membrane potential stimulates V-ATPase to pump protons into the compartment (2). Acidification results in the attraction of membrane permeant weak bases (3), which become protonated, and trapped within the compartment (4). The resulting osmotic imbalance leads to the inf lux of water (5), and vacuole swelling. Adapted f rom Tombola *et al*., (1999a).

internalisation of VacA anion channels into late endosomal compartments leads to the influx of chloride ions, reducing the inside-positive membrane potential. This stimulates V-ATPase to increase the proton concentration inside the vacuole, lowering the internal pH. Membrane permeant weak bases enter the vacuole, become trapped owing to their protonation and accumulate. The resulting osmotic imbalance would lead to water influx and vacuole swelling. This model provides a good explanation for why weak bases and an active V-ATPase are required for VacA-induced vacuolation. Genisset *et al* (2007) have recently tested this model using an isolated endosome system. Addition of VacA caused an anion-dependent acidification of the endosomal compartment, consistent with enhanced v-ATPase activity, and this led to an increase in endosome size. Another recent study has identified point mutations within the p33 domain of VacA which block vacuolating activity without affecting oligomerisation of the toxin, or its binding, internalisation or ion channel formation (McClain *et al.*, 2006). The ion channels formed by these mutated forms of VacA had the same anion selectivity as wild type VacA, although differences in channel formation rate were noted at a

positive, but not negative, membrane potential. These observations raise the possibility that anion-selective channel formation is required but not sufficient for vacuolating activity.

The swelling of late-endosomal/lysosomal compartments induced by VacA channel activity means that additional membrane is required, and this most likely derives from vesicular fusion. The fusion of biological membranes is controlled by a complex machinery, the core of which are the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins, small membrane proteins with cytoplasmic amphipathic helical motifs which participate in coiled coil interactions (reviewed in Hay, 2001). The homotypic fusion of late endosomes involves a complex of the SNARE motif proteins syntaxin 7, syntaxin 8, vti 1b and endobrevin (Antonin *et al.*, 2000; Mullock *et al.*, 2000; Nakamura *et al.*, 2000; Ward *et al.*, 2000). Using a dominant negative syntaxin 7 mutant and anti-syntaxin 7 antibodies, de Bernard *et al.* (2002) reported that late endosomal fusion mediated by syntaxin 7 is not required for VacA-induced vacuolation of HeLa cells, and suggest that the vacuolar membrane may derive from the fusion of internal membranes present in this organelle with its cytosolic membrane. In contrast, transfecting AGS cells with a syntaxin 7 dominant negative mutant did inhibit vacuolation in this cell line, and VacA intoxication was shown to increase the syntaxin 7 expression in untransfected AGS cells (Suzuki *et al.*, 2003).

Dynamin, a large GTP-binding protein, catalyses several steps in vesicle formation and trafficking, and exists in three isoforms with dynamin-2 being ubiquitously expressed (reviewed in Kirchhausen, 1998). VacA-induced vacuoles have been shown to contain dynamin-2 (Suzuki *et al.*, 2001), although its involvement in vacuolation is unclear. Expression of a dominant negative mutant of dynamin-1, which also inhibits dynamin-2 function, inhibited vacuolation of HeLa and MKN28 cells without affecting VacA internalisation (Suzuki *et al.*, 2001). However, Ricci et al (2000) report that a dominant negative dynamin-2 mutant did not affect VacA-induced vacuolation of HEp-2 cells. It has also been suggested that VacA may indirectly inhibit the phophatidylinositol kinase activity of PIKfyve triggering vacuolation, as active PIKfyve inhibits VacA-induced vacuolation while inactivation of PIKfyve in the absence of VacA results in intracellular vacuolation resembling that induced by the toxin (Ikonomov *et al.*, 2002).

1.6 Other functional roles of VacA

While the induction of cytoplasmic vacuolation was the first described effect of VacA intoxication of epithelial cells *in vitro*, several other functions have been ascribed to this toxin. These include other effects on epithelial cells and possible modulation of the host immune response. Many of these VacA-related effects arise from the anion channel activity of the toxin, but some may involve signalling mechanisms triggered through VacA binding to cell surface receptors.

1.6.1 Increasing plasma membrane permeability

The ability of VacA to form anion-selective channels within biological membranes has been discussed in relation to vacuolation. However, their role in causing osmotic imbalance of endosomal compartments leading to vacuolation occurs after they have been internalised through endocytosis. In contrast, the main function of VacA channels may be to allow the release of essential nutrients from the apical surface of the gastric epithelium before their subsequent internalisation, promoting growth of *H. pylori* localised deep within the mucus layer close to the epithelial surface. Indeed, VacA channels have been shown to increase the permeability of planar lipid bilayers to bicarbonate ions and pyruvate (Tombola *et al.*, 1999a), the latter being an essential requirement for *H. pylori* growth (Testerman *et al.*, 2006). It has also been shown that VacA channels permeabilise the plasma membrane of cultured epithelial cells to urea, suggesting that the toxin may aid colonisation by acting as a urea permease *in vivo*, thereby increasing the availability of this important urease substrate required for protection against low pH (Tombola *et al.*, 2001a). Indeed, while isogenic *vacA* null mutant strains have been shown to colonise gnotobiotic piglets (Eaton *et al.*, 1997) and Mongolian gerbils (Wirth *et al.*, 1998), competition experiments with a wild type strain in a mouse model suggest the toxin may have a role in initial colonisation (Salama *et al.*, 2001).

1.6.2 Effect on transepithelial resistance

The addition of purified VacA to polarised monolayers of MDCK cells , T84 (human colonic epithelial cells) and epH4 (murine mammary gland) cells results in a decrease in trans-epithelial resistance (TER) (Papini *et al.*, 1998). The effect is dependent on the concentration

of VacA and is greatly enhanced by acid-activation. VacA bound to the *H. pylori* surface has also been shown to induce a decrease in TER of polarised MDCK cells (Papini *et al.*, 1998; Pelicic *et al.*, 1999). VacA-induced decreases in TER appear to be independent of the vacuolating activity of the toxin as polarised MDCK monolayers did not vacuolate in the presence of VacA, unlike nonpolarised cells, and the effect was not inhibited by bafilomycin A1, which inhibts V-ATPase activity essential for VacA-induced vacuolation (Papini *et al.*, 1998). Furthermore, a similar effect on TER was observed for an s1/m2 strain which lacked the ability to vacuolate sparse MDCK cells (Pelicic *et al.*, 1999). VacA does not cause major disruption of polarised monolayers as following treatment TER does not fall below 1000 Ω x cm^2 which is still indicative of good tight junction integrity, and there is no effect on the amount or localisation of the tight junction proteins ZO-1, occludin and cingulin, or E-cadherin, a protein associated to the adherens junction (Papini *et al.*, 1998). However, some weakening of the tight junctions most likely occurs since VacA-treated monolayers show an increased permeability to mannitol, a membrane impermeable sugar used as a marker for the paracellular pathway (Papini *et al.*, 1998; Pelicic *et al.*, 1999). Weaking of the epithelial tight junctions *in vivo* may allow the release of nutients promoting *H. pylori* growth, and in this respect VacA-induced increased permeability of MDCK monolayers to Fe^{3+} and Ni^{2+} ions has been noted (Papini *et al.*, 1998), both important components for *H. pylori* colonisation. Interestingly, one of the identified VacA receptors, RPTPI3, has been shown to interact with the scaffolding protein MAGI-3 (membrane-associated guanylate kinase with inverted orientation 3) located at epithelial tight junctions, where it has been demonstrated to link RPTPI3 with tyrosine-phosphorylated protein substrates (Adamsky *et al.*, 2003). This may allow VacA, as a ligand for RPTPI3, to influence cell signalling pathways regulating tight junction integrity. Ligand binding to RPTPI3 has also been shown to affect the tyrosine phosphorylation of I3-catenin (Meng *et al.*, 2000), a molecule also implicated in tight junction formation (Rajasekaran *et al.*, 1996).

1.6.3 Induction of mitochondrial damage and apoptosis

Several lines of evidence suggest that VacA is able to target the mitochondria of epithelial cells. Transfection of HEp-2 cells with DNA encoding the VacA p33 and p55 fragments

showed that the p33 VacA domain localised to mitochondria while the p55 domain remained cytosolic (Galmiche *et al.*, 2000). The same authors also showed that full sized VacA, as well as the p33 fragment, could translocate into purified yeast mitochondria *in vitro*. More recently, both VacA domains have been found to enter the intermembrane space of mitochondria purified from mice (Foo *et al.*, 2010). VacA has also been shown to localise to mitochondria when delivered externally to HeLa and AGS cells (Willhite and Blanke, 2004), although Yamasaki *et al.* (2006) reported that the majority of the toxin remained associated with vacuoles within AZ-521 gastric cells. Interestingly, this latter cell line is very sensitive to VacA-induced cell death, but the mechanism is characteristic of programmed necrosis rather than apoptosis (Radin *et al.*, 2011). The hydrophobic N-terminus of the p33 domain appears to be the signal for mitochondrial localisation (Domanska *et al.*, 2010). VacA targeting of mitochondria results in disruptive effects on energy metabolism including a decrease in mitochondrial membrane potential, a reduction in cellular ATP levels and inhibited oxygen consumption, as demonstrated in AZ-521 gastric epithelial cells (Kimura *et al.*, 1999). VacA has also been shown to induce the release of cytochrome *c* from mitochondria into the cytosol (Galmiche *et al.*, 2000; Willhite *et al.*, 2003; Willhite and Blanke, 2004). These effects of VacA on mitochondrial function are not blocked by bafilomycin A1 implying they are independent of vacuolating activity (Kimura *et al.*, 1999; Willhite *et al.*, 2003). However, nonvacuolating forms of the toxin with altered anion channel activity, such as s2-type VacA and VacA A6-27, do not reduce mitochondrial membrane potential or induce cytochrome *c* release, implying that channel activity is also required for these effects (Cover *et al.*, 2003; Willhite *et al.*, 2003; Willhite and Blanke, 2004). Furthermore, VacA-induced cytochrome *c* release is blocked by chloride channel inhibitors to the same extent as vacuolation (Willhite *et al.*, 2003). VacA-induced cytochrome *c* release occurs after a reduction in mitochondrial membrane potential, and requires higher toxin concentrations (Willhite and Blanke, 2004), which implies that VacA primarily affects inner mitochondrial membrane permeability, possibly through channel formation. Consistent with this view, VacA is able to directly reduce the membrane potential of purified mitochondria, but not induce cytochrome *c* release, the latter requiring the activation of proapoptotic Bcl-2-family proteins Bax and Bak (Yamasaki *et al.*, 2006). The release of cytochrome *c* from mitochondria is an important initiator of apoptosis, and it has been shown

that VacA-induced cytochrome *c* release leads to activation of procaspase 3, and the subsequent induction of apoptosis in AGS and HeLa cells (Cho *et al.*, 2003; Cover *et al.*, 2003; Galmiche *et al.*, 2000; Kuck *et al.*, 2001). Finally, recent work has shown that purified VacA p33 domain is able to form hexameric and dodecameric complexes, and form anion channels within artificial lipid bilayers in the absence of the p55 domain, suggesting that the effects of VacA on mitochondria may be accounted for by just the targeting of this domain to the organelle (Domanska *et al.*, 2010).

1.6.4 Immunosuppressive and proinflammatory effects

It has been suggested that VacA may play an immunosuppressive role during infection by blocking T cell activation (Boncristiano *et al.*, 2003; Gebert *et al.*, 2003). The toxin has been shown to bind to Jurkat human leukemic T cells and human peripheral blood lymphocytes *in vitro*, inhibiting their proliferation by inducing cell cycle arrest at the G1 phase. The mechanism deduced in Jurkat cells involves preventing translocation of the NFAT (nuclear factor of activated T cells) transcription factor to the nucleus, thus suppressing the expression of cytokines such as IL-2, essential for T cell proliferation (Boncristiano *et al.*, 2003; Gebert *et al.*, 2003). Cytoplasmic NFAT translocates to the nucleus upon dephosphorylation by calcineurin, which is itself activated by the cellular influx of Ca^{2+} ions triggered by T cell antigen stimulation. The influx of calcium ions into T cells is driven by membrane potential via Ca^{2+} release-activated Ca^{2+} (CRAC) channels. The anion channel properties of VacA appear to interfere with this process, presumably by depolarising the plasma membrane (Boncristiano *et al.*, 2003), hence s2 forms of the toxin with altered channel properties do not reduce the IL-2 production of Jurkat cells (Algood *et al.*, 2007). There is also evidence for a second, channel-independent mechanism of VacA induced inhibition of T cell activation, since the p55 fragment of VacA, which binds to cells but is not internalised or able to form channels, still blocks expression of the T cell activation marker CD69 (Boncristiano *et al.*, 2003). This second mechanism appears to involve the VacA-induced activation of stress-activated kinase p38 but not extracellular signal-regulated kinase (ERK)1/2, and is presumably triggered by VacA binding to a cell surface receptor as activation of the nucleotide exchange factor Vav and serine/threonine kinases MKK3/6 also occurs (Boncristiano *et al.*, 2003). Vav activation

also leads to increased activity of the small GTPase Rac resulting in actin reorganisation. The proliferation of activated primary human CD4+ T cells has also been shown to be blocked by VacA, however, unlike Jurkat T cells, NFAT translocated to the nucleus as normal and IL-2 secretion was not reduced (Sundrud *et al.*, 2004). Instead, the toxin suppressed IL-2-dependent cell cycle progression by a mechanism which also appears to be dependent on its anionic channel forming properties. In contrast to these studies, Gerhard *et al.* (2005) have found that *H. pylori*-induced cell cycle arrest of human peripheral blood lymphocytes did not involve VacA, but another secreted protein later identified as ~-glutamyltranspeptidase (Schmees *et al.*, 2007).

VacA may also indirectly inhibit T cell proliferation by interfering with antigen presentation. Molinari *et al.* (1998b) have shown that VacA treatment significantly reduces the proteolytic processing of tetanus toxin in Epstein-Barr virus-transformed B cells. Ii-dependent antigen presentation to CD4+ T cells was affected, but not the Ii-independent pathway. The former pathway relies on the proteolytic processing of antigens within antigen-processing compartments, and their loading onto newly synthesised major histocompatibility complex (MHC) class II molecules, while the Ii-independent route utilises epitopes generated in less acidic early endosomal compartments and recycled MHC class II. No direct effects on antigen loading of MHC class II molecules or their surface presentation was observed (Molinari *et al.*, 1998b). Antigen-processing compartments are similar to late endosomes, and their low pH is necessary for antigen denaturation and protease activity. VacA has been shown to partially neutralise endosomal and lysosomal compartments in HeLa cells inhibiting the degradation of intracellular epidermal growth factor (Satin *et al.*, 1997), and a similar mechanism most likely accounts for the toxin's effect on antigen processing.

In addition to the immunosuppressive actions described above, the vacuolating cytotoxin has also been shown to have proinflammatory effects on gastric mucosal mast cells. Oral administration of purified toxin to mice caused marked infiltration of mast cells and mononuclear cells into the mucosal epithelium (Supajatura *et al.*, 2002). Murine bone marrow-derived mast cells (BMMCs) showed both a chemotactic attraction to VacA, and bound the toxin *in vitro*. VacA treatment of BBMCs induced the secretion of proinflammatory cytokines including tumor necrosis factor (TNF) a, macrophage-inflammatory protein-1a, IL-1b, IL-6, IL-

10 and IL-13 in a dose-dependent manner. Similarly, VacA stimulation of the rat basophilic leukaemia mast cell line RBL-2H3 upregulated TNF-a mRNA and protein levels in a calcium-dependent manner (de Bernard *et al.*, 2005). The mechanism appears to involve VacA-induced rapid ocillations in cytosolic calcium levels, the calcium ions originating from intracellular stores such as the endoplasmic reticulum. Degranulation of the mast cells was observed for VacA-treated RBL-2H3 cells (de Bernard *et al.*, 2005), but not mouse BMMCs (Supajatura *et al.*, 2002). VacA has also been shown to have proinflammatory effects on T-cells, in contrast to earlier studies describing immunosuppressive effects as discussed above. Treatment of Jurkat cells with VacA results in activation of NFKB signalling, leading to the upregulation of many genes, including the proinflammatory cytokine IL-8 (Takeshima *et al.*, 2009). One reason for this discrepancy may be that immunosuppressive effects were seen when polyclonally activated T-cells were treated with VacA, while the study showing a proinflammatory response used unactivated T-cells (Takeshima *et al.*, 2009).

1.6.5 Activation of cell-signalling pathways

There is some evidence that VacA is able to activate the MAPKs p38 and ERK1/2 *in vitro*. The VacA-induced activation of the stress-activated kinase p38 pathway in Jurkat cells (Boncristiano *et al.*, 2003) has already been described, and in the same study, VacA-induced activation of p38 was observed in neutrophils and macrophages leading to increased expression of COX-2, an enzyme catalysing the production of prostaglandins and other prostanoids. VacA has also been shown to activate the p38 and ERK1/2 pathways in AZ-521 gastric cells leading to phosphorylation of activating transcription factor 2, and subsequent increased COX-2 expression and prostaglandin E_2 (PGE_2) synthesis (Hisatsune *et al.*, 2007; Nakayama *et al.*, 2004). Increased COX-2 expression plays an important role in gastrointestinal tumorigenesis, including gastric adenocarcinoma, through several mechanisms including promoting proliferation and inhibiting apoptosis (reviewed in Fujimura *et al.*, 2006). Furthermore, COX-2 has been shown to suppress the production of Th1 cytokines in *H. pylori*-stimulated human peripheral blood mononuclear cells through its effect on PGE_2 synthesis (Meyer *et al.*, 2003), and in gastric biopsies from *H. pylori*-infected patients (Pellicano *et al.*, 2007). The phosphorylation of p38 induced by VacA treatment of

AZ-521 cells is independent of other toxin effects such as vacuolation, decreased mitochondrial membrane potential and cytochrome *c* release (Nakayama *et al.*, 2004). Additionally, broth culture supernatants from a wild type *H. pylori* strain, but not an isogenic *vacA* mutant, induced increased vascular endothelial growth factor (VEGF) mRNA and protein expression in MKN-28 gastric cells by a mechanism which involved the activation of ERK, expression of COX-2, and required epidermal growth factor receptor tyrosine kinase activity (Caputo *et al.*, 2003). VEGF is an important angiogenic cytokine involved in the repair of damaged mucosa, but also plays a role in tumor vascularisation (Veikkola and Alitalo, 1999).

1.7 Naturally occurring *vacA* polymorphism

When the vacuolating activity of *H. pylori* broth culture supernatants was first described it was noted that only about half of the strains studied produced this effect (Leunk *et al.*, 1988). Hybridisation and polymerase chain reaction experiments showed that these nontoxigenic strains still possessed the *vacA* gene, although genetic diversity was apparent (Cover *et al.*, 1994; Garner and Cover, 1995; Phadnis *et al.*, 1994; Schmitt and Haas, 1994; Telford *et al.*, 1994). Sequence analysis of *vacA* from a nontoxigenic strain confirmed that nucleotide differences existed throughout the gene, and highlighted two regions of greatest sequence diversity: the signal region, encoding part of the signal peptide and the N-terminus of the mature protein (which may be type s1 or s2) and the mid region, a 0.73 kb segment encoding part of the p55 domain (type m1 or m2)(Atherton *et al.*, 1995)(see Figure 2D). Two subtypes of the s1 signal region were originally described (s1a and s1b), and a further, s1c type has been described in strains isolated from East Asia (van Doorn *et al.*, 1998b). Similarly, an m2b mid region subtype (the original referred to as m2a) has been described for East Asian strains (van Doorn *et al.*, 1998b), and an m1a variant identified in Germany (Strobel *et al.*, 1998). Natural mosaicism occurs such that all combinations of signal and mid region type exist (Atherton *et al.*, 1995), although the s2/m1 form of the toxin is rarely found naturally (Kidd *et al.*, 1999; Letley *et al.*, 1999 [see Chapter 2]; Morales-Espinosa *et al.*, 1999), despite artificially constructed s2/m1 strains growing equally well *in vitro* (Letley *et al.*, 2003 [see Chapter 4]; McClain *et al.*, 2001b).

1.7.1 The association between *vacA* alleles and toxin activity

Functionally, s1-type strains are associated with vacuolating activity *in vitro*, while s2-type strains are invariably nonvacuolating (Atherton *et al.*, 1995; Rudi *et al.*, 1998). The s1-form of the toxin has been extensively studied, and collective evidence has shown that the hydrophobic N-terminus of the toxin plays a vital role in the vacuolation process. Truncations, internal deletions and even single point mutations within this region have been shown to have dominant negative effects on the vacuolating activity of the toxin either expressed intracellularly or delivered externally to cells (de Bernard *et al.*, 1998a; McClain *et al.*, 2006; Vinion-Dubiel *et al.*, 1999; Ye *et al.*, 1999; Ye and Blanke, 2000). Characterisation of one such mutant, VacA (A6-27), showed that this nonvacuolating form of the toxin still assembled into oligomeric structures similar to those formed by wild type VacA, bound and entered HeLa cells, and formed ion conductive channels in planar lipid bilayers (Vinion-Dubiel *et al.*, 1999). However, ion channels formed more slowly and were less anion-selective, suggesting that the properties of the VacA ion channel are critical for VacA-induced vacuolation.

The hydrophobic region located near the N-terminus of s1-type VacA, and evidently important for vacuolating activity, is also present in the nonvacuolating, s2-form of the toxin. However, this region is preceeded by a hydrophilic N-terminal extension of 12 amino acid residues, unique to s2-type strains, which results from differences in signal sequence processing site position coupled with an insertion encoding an additional 9 amino acids (Atherton *et al.*, 1995). It has been shown that the addition of this hydrophilic extension to the s1-form of the toxin completely blocks vacuolating activity, while its removal from s2-type VacA, resulting in an s1-like N-terminus, restores vacuolating activity to an otherwise nonvacuolating strain (Letley and Atherton, 2000 [see Chapter 3]; Letley *et al.*, 2003 [see Chapter 4]; McClain *et al.*, 2001b). The chimeric s2/m1 form of VacA resulting from the addition of the hydrophilic s2-specific extension to s1/m1 VacA formed channels in planar lipid bilayers at a significantly slower rate than s1/m1 VacA, but they still showed the same anion-selectivity (McClain *et al.*, 2001b). This implies that the rate of channel formation is an important factor in the mechanism of vacuolation. Thus, differences in VacA signal region type determine the vacuolating activity of the toxin by modifying its channel forming properties. Signal region type may also act as a marker for differences in the promoter region of *vacA*,

since exchange experiments have shown this region to also determine the level of toxin produced (Letley *et al.*, 2003 [see Chapter 4]).

Polymorphic differences in the mid-region of VacA determine the target cell specificity of vacuolation. For example, using naturally occuring s1/m1 and s1/m2 strains (Pagliaccia *et al.*, 1998), as well as artificially constructed, isogenic chimeric strains (Ji *et al.*, 2000; Letley *et al.*, 2003 [Chapter 4]), it has been shown that s1/m1 VacA vacuolates both HeLa and RK13 cells while s1/m2 VacA vacuolates RK13, but not HeLa cells. Importantly, VacA from one s1/m2 strain has been shown to vacuolate primary cultured human gastric cells (Pagliaccia *et al.*, 1998). Within the m1-type mid region, the first 35 amino acids have been shown to be essential for vacuolating HeLa cells (Ji *et al.*, 2000). This region differs to the m2-form mainly by the lack of a 25 amino acid insertion present in the later. The following 113 residues are also important in determining the level of HeLa cell-specific vacuolating activity observed (Ji *et al.*, 2000). Tombola *et al.* (2001b) observed that the channel properties of an s1/m1 and an s1/m2 toxin were very similar, and it is unlikely that subtle differences account for the lack of activity in a HeLa cell vacuolation assay as intracellular expression of s1/m2 VacA in HeLa cells results in vacuolation (Pagliaccia *et al.*, 1998). Instead, the cell specificity of the mid region can be attributed to differences in toxin binding to different target cells. Pagliaccia et al (1998) first showed that s1/m2 VacA bound weakly to HeLa cells, unlike the m1 form, or the binding of either form to RK13 cells. A later study identified two VacA cell binding patterns: a high affinity, m1-specific binding site and a lower affinity receptor able to bind both m1 and m2 forms (Wang *et al.*, 2001). The binding of purified m2-type VacA to both RPTP$_{CL}$ and RPTP$_{I3}$ receptors in AZ-521 gastric cells, and to RPTP$_{CL}$ in G401 cells, can be increased by acid or alkali activation (De Guzman *et al.*, 2005). However, unlike the m1 form of the toxin, m2-type VacA still bound poorly to RPTP$_{CL}$ in HeLa cells, even when acid activated. This cell specificity in VacA binding appears to result from differences in glycosylation of RPTP$_{CL}$ in HeLa cells, and is independent of the RPTP$_{I3}$ receptor (De Guzman *et al.*, 2005; Skibinski *et al.*, 2006). The higher sensitivity of RK13 cells to the m1 form of VacA compared with HeLa cells most likely reflects the higher expression of RPTP$_{CL}$ in RK13 (and AGS) cells than HeLa cells (De Guzman *et al.*, 2005; Pagliaccia *et al.*, 1998). Thus differences in the mid region of the toxin

coupled with alterations in glycosylation and expression level of the $RPTP_{CL}$ VacA receptor appear to determine the susceptibility of cells to VacA-induced vacuolation.

Recently, Rhead *et al.* (2007)[Chapter 6] described a new polymorphic determinant of VacA toxicity called the intermediate region, which may be type i1 or i2. This region is located between the previously described signal and mid regions within the region encoding the p33 domain of the toxin (see Figure 2D). While VacA s1/m1-type strains were nearly always type i1 and s2/m2 type strains were always i2-type, s1/m2-type strains varied in their i-region status and this correlated with their ability to vacuolate RK13 cells: s1/i1/m2 strains induced vacuolation while s1/i2/m2 strains were nonvacuolating. Site-directed mutagenesis experiments confirmed that i-region type determined vacuolating activity in an s1/m2 strain background, and showed that this region influenced the cell-specificity of the toxin in the context of an s1/m1-type strain, implying that it may be involved in toxin binding in a similar manner to the mid region. The amino terminal 422 residues of VacA represent the minimal domain required for vacuolating activity when the toxin is expressed intracellularly (Ye *et al.*, 1999). This domain includes the signal and intermediate regions, but not the mid region, and therefore differences in the intermediate region may instead influence the ability of the toxin to oligomerise or alter its anion channel activity.

1.7.2 The geographical distribution of *vacA* allelic types, and their association with gastric disease

Since the identification of *H. pylori* vacuolating activity, studies have focused on its association with upper gastrointestinal disease. Early studies relied on HeLa cell vacuolation assays to determine toxin activity, but as these cells are unable to bind active s1/m2 forms of the toxin as discussed earlier, not all potentially pathogenic strains would have been identified. However, despite this limitation, strains producing vacuolating activity *in vitro* have been associated with the presence of peptic ulcer disease in infected patients (Atherton *et al.*, 1995; Figura *et al.*, 1989; Rautelin *et al.*, 1994; Tee *et al.*, 1995; Zhang *et al.*, 1996). Patient serum responses to VacA have also been used to evaluate the role of the toxin in *H. pylori* pathogenesis (Donati *et al.*, 1997; Shimoyama *et al.*, 1999; Takata *et al.*, 1998a; Yamaoka *et al.*, 1999). However, as the majority of strains express the toxin, and antibody responses to

one allelic form of the toxin cross-react, albeit less strongly, to other forms (Ghose ***et al.***, 2007; Pérez-Pérez ***et al.***, 1999), this approach has been largely unsuccessful in associating VacA toxicity with disease.

A more successful approach has been the association of specific ***vacA*** allelic forms with gastric disease using simple PCR-based typing systems (Atherton ***et al.***, 1995; Atherton ***et al.***, 1999a) and a reverse hybridisation line probe assay (van Doorn ***et al.***, 1998a; van Doorn ***et al.***, 1998b). These methods have led to many studies correlating ***vacA*** allelic type and upper gastrointestinal disease. In many populations where ***vacA*** diversity has been found, most notably Western Europe and the USA, ***vacA*** s1/m1-type strains have been associated with duodenal and gastric ulceration (Atherton ***et al.***, 1995; Atherton ***et al.***, 1997; Figueiredo ***et al.***, 2001; Rudi ***et al.***, 1998; Strobel ***et al.***, 1998) and gastric adenocarcinoma (Basso ***et al.***, 1998; Evans ***et al.***, 1998; Figueiredo ***et al.***, 2001; Kidd ***et al.***, 1999; Miehlke ***et al.***, 2000). However, even in populations where ***vacA*** allelic type is quite homogeneous, there may be a connection with ***H. pylori***-related disease. For example, the ***vacA*** s1/m1-type allele associated with upper gastrointestinal disease in western populations is the predominant allele found in Japan, a country with a high incidence of gastric cancer (Ito ***et al.***, 1997; Kodama ***et al.***, 1999; Sadakane ***et al.***, 1999; Shimoyama ***et al.***, 1998; Takata ***et al.***, 1998b; Zhou ***et al.***, 2004). The reported association between gastric adenocarcinoma and infection with ***vacA*** s1/m1-type strains is not absolute as some of the cancer patients in these studies are infected with s1/m2-type strains. Instead, the recently described ***vacA*** i-region may provide a better marker of gastric adenocarcinoma risk. As described earlier, s1/m2 strains vary in their i-region type and this determines their ability to vacuolate RK13 epithelial cells ***in vitro*** (Rhead ***et al.***, 2007). A case-control study of ***vacA*** allelic type and gastric adenocarcinoma among patients from Iran found that while s1, m1 and i1 alleles, and ***cagA*** status, were all associated with gastric adenocarcinoma, only i-region status was independent of the other alleles studied. As all s1/m1 strains, and those s1/m2 strains which displayed vacuolating activity ***in vitro***, were found to be i1-type, determining i-region type may be sufficient for identifying all pathogenic forms of VacA.

1.8 Aims

The overall aim of the published papers presented in this thesis was to characterise natural *vacA* polymorphism, and how it affects the vacuolating activity of the secreted cytotoxin. The more specific aims of each paper were as follows:

Paper 1 (Chapter 2)

vacA allelic variation had been previously characterised among *H. pylori* strains isolated from USA, Europe and East Asia, but not among isolates from any African countries, where *H. pylori* prevalence is high, but the incidence of gastric disease is reportedly low. We aimed to determine what *vacA* alleles were present among a small number of strains isolated from South Africa, and compare this with previously studied strains from USA and East Asia.

Papers 2 and 3 (Chapters 3 and 4)

The diverse *vacA* signal and mid-regions have been previously described, and the s1/m1 form shown to be associated with vacuolating activity *in vitro*, and with increased risk of peptic ulcer disease and gastric adenocarcinoma. We aimed to:

- Assess whether signal region type determined the amount of cytotoxin produced.
- Determine the role of signal and mid-region polymorphic differences on the vacuolating activity of the cytotoxin.

Paper 4 (Chapter 5)

All forms of VacA contain a conserved pair of cysteine residues spaced 11 amino acids apart, and located near the C-terminus of the mature, secreted protein. Several other autotransporters also contain a single pair of cysteine residues with similar spacing, suggesting they may be required for autotransporter secretion. We aimed to determine whether these cysteine residues were required for VacA secretion.

Paper 5 (Chapter 6)

The construction of *vacA* hybrid strains in Paper 3 (Chapter 4) led to the fortuitous discovery of a new polymorphic region of *vacA* which appeared to affect vacuolating activity. We aimed to:

- Define the exact location of this new determinant of vacuolating activity.
- Confirm that this new, intermediate region determined vacuolating activity in different strain backgrounds.
- Assess whether polymorphic differences in this region were common in other *H. pylori* strains.
- Design and evaluate a PCR-based typing system to quickly determine the intermediate region type of *H. pylori* clinical isolates.
- Evaluate whether intermediate region type was a risk factor for gastric disease, and compare it to other *vacA* allelic markers of disease risk.

CHAPTER 2

ALLELIC DIVERSITY OF THE *Helicobacter pylori* VACUOLATING CYTOTOXIN GENE IN SOUTH AFRICA: RARITY OF THE *VACA* s1a GENOTYPE AND NATURAL OCCURRENCE OF AN s2/m1 ALLELE

Letley, D. P., A. Lastovica, J. A. Louw, C. J. Hawkey and

J. C. Atherton (1999)

Journal of Clinical Microbiology **37**:1203-1205

JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1999, p. 1203-1205 Vol. 37, No. 4
0095-1137/99/SO4.00+0

Allelic Diversity of the *Helicobacter pylori* Vacuolating Cytotoxin Gene in South Africa: Rarity of the *vacA* s1a Genotype and Natural Occurrence of an s2/m1 Allele

D. P. LETLEY,'•2 A. LASTOVICA,3•4 J. A. LOUW,4'5 C. J. HAWKEY,' AND J. C. ATHERTON[L2]*

Division of Gastroenterology' and Institute of Infections and Immunity,' University of Nottingham. Nottingham.
United Kingdom, and Red Cross Children's Hospital,' Groote Schuur Hospital[s] and University of Cape Town,' Cape Town, South Africa

Received 13 October 1998/Returned for modification 23 November 1998/Accepted 1] January 1999

We **describe the rarity** of *Helicohacier pylori* **strains of vacuolating cytotoxin type sla (the type most commonly associated with peptic ulceration in the United States) among black and mixed-race South Africans. We also provide the first description of a naturally occurring strain with the *vacA* allelic structure s2/ml.**

Helicobacter pylori* colonizes the human gastric mucosa, leading to chronic superficial gastritis, and is an important risk factor for peptic ulceration, gastric adenocarcinoma, and gastric lymphoma. Two virulence determinants have been described: a 40-kb pathogenicity island for which** the gene ***cagA **(cytotoxin-associated gene A) is a marker (5) and** a secreted **cytotoxin, VacA. The** cytotoxin causes vacuolation of epithelial cells **in vitro (12) and induces epithelial cell damage and mucosa] ulceration when administered orally to mice (14). Although fewer than 50% of H *pylori* clinical isolates from the United** States produce HeLa cell-vacuolating activity, the gene ***vacA*, which encodes the cytotoxin, has been found in all strains studied** (6). However, ***vacA* alleles vary between toxigenic (Tox+) and nontoxigenic (Tox-) strains, the differences being most marked in the region encoding the signal sequence and *the*** mid-region **of the gene (2). *vacA* alleles** of strains from the United **States, Europe, and Asia are mosaics consisting of any combination of the three signal sequence types (sla, s lb, or s2) and two mid-region types (ml or m2), with the exception of s2/m1 (2, 3). The mosaic structure of *vacA* could be explained by stepwise acquisition of stretches** of **DNA as single isolated events followed by clonal expansion or by acquisition of DNA and subsequent recombination between *vacA* alleles among *H. pylori* strains. Several lines of evidence, namely multilocus enzyme electrophoresis (7, 8) and** the **use** of genetic **markers (13), suggest that recombination is frequent in *H. pylori.* If recombination occurs within *vacA, it* is unclear why *vacA* s2/m1 alleles have not been found, especially** since we have **introduced an artificially constructed *s21m1* allele into two strains** and have shown them to be viable in vitro (11). In the United States, strains with ***vacA*** s la alleles arc associated with peptic ulceration more frequently than those with slb or s2 alleles (4). ***vacA* diversity among *H. pylori*** strains from **South Africa** has not **previously been studied but may be clinically important because the scarcity of pathogenic strains could explain the "African enigma" of high levels of *H. pylori* infection but** relatively low levels of peptic ulcer disease and gastric adenocar-cinoma in Africa **(10). For** this reason we studied *vacA* **allele diversity among South African *H. pylori*** isolates.

Corresponding author. Mailing address: Division of Gastroenterology, **Queen's Medical Centre, University Hospital,** Clifton Boulevard, Nottingham NG7 2UH, United Kingdom. Phone: (44) 115 9249924. Fax: (44) 115 9422232. E-mail: John.Atherton@nottingham.ac.uk.

We examined single-colony ***H. pylori* isolates from 16** South African patients having a median age of 36 years (range, 20 to 63 years). Fifteen patients were black or of mixed race, and 1 was caucasian; 11 were male, 11 had active duodenal ulcers, and ***5* were asymptomatic without ulcers. None were taking nonsteroidal anti-inflammatory drugs. For three patients, two morphologically distinct colonies were isolated and examined separately. Chromosomal DNA was extracted from 48-h plate cultures of each strain by a previously described guanidine thiocyanate-EDTA-Sarkosyl lysis method (1). For each isolate, the *vacA* signal sequence and mid-region were characterized by PCR as previously described** (2). **The presence or absence of *cagA* was determined for each isolate by DNA hybridization: samples of each genomic DNA** were **applied to a nylon membrane and hybridized with a 349-bp digoxigenin-labelled *cagA* probe derived from *H. pylori* 84183 (2).**

***vacA* and *cagA* genotypes were** successfully and fully determined for each isolate; results arc shown in Table 1. For the three patients from whom two morphologically distinct colonies were isolated, both colonies showed the same *vacA* and ***cagA* genotype, and for clarity, only one isolate has been included in Table 1. A single *vacA* sla/m I** strain was **found; it was isolated from the one caucasian patient in the study. Of the 15 black or mixed-race South Africans in this study, 10 had *vacA* slbiml isolates, 4 had slb/m2 isolates, and 1 had s2/m1 isolates (two isolates from the same patient).**

We compared the *vacA* and *cagA* genotypes of South African strains from this study with those of previously reported strains from the United States (2, 4) and Asia (3) (Fig. 1). There are sinking geographical differences. The absence of ***the vacA*** sla allele among isolates from black and mixed-race subjects from South Africa contrasts with the finding of sla alleles among all Asian strains studied ***(P < 10 '°,*** Fisher's exact test). The prevalence of type sla ***vacA*** alleles among South African strains was also significantly less than that among strains from the United States, where a more even **spread** of strains with the **three different** signal types was found (34% sla; ***P* < 0.01). These comparative data between strains from different continents arc potentially influenced by disease state, as there is a recognized link between the *vacA*** sla genotype and peptic ulceration in the United States (4). However, if this is controlled for by considering only patients with peptic ulcers, the results are even more striking. Among such patients, none of 10 black or mixed-race South Africans had strains with ***vacA sla*** alleles, which is less than the 100% of 14

TABLE 1. *vacA* and *ragA* genotypes and vacuolating activity of ***H. pylori*** isolates from South Africa

N. pylori isolate	Clinical disease of infected patient'	rac.4 genotype		Vacuolating activity`	*ragA* genotype
		Signal	Mid-region		
HP218	DU	slb	rn2		
HP220	DU	slb	ml		
HP233	DU	slb	m2		
HP442	DU	slb	ml	ND	
HP458`4	DU	slb	ml		
HP460	DU	slb	ml		
HP464	DU	slb		ND	
HP465``	DU	s2			
HP466	DU	sib	ml		
HP467	No ulcer	sib	ml	ND	
HP468	DU	sib	n12		
HP469	DU	slb	m2		
HP501	No ulcer	sib		ND	
HP508	No ulcer	slb	m t		
11 P517	No ulcer	slb	m]		
FI P548	No ulcer	slb	ml		

DU. duodenal ulcer. No ulcer patients were asymptomatic.

'*H. pylori* isolates were delined as producing vacuolating activity (+) when >80% vacuolation of AGS cells was observed rifler application of unconcentrated broth culture supernatant [diluted twofold or more). **ND. nol** assayed. A second, morphologically distinct colony from the same original culture plate was characterized separately and found to he the same (data mit shown). ' This isolate wax from the one eaucasian patient in !he study, All other strains were isolated. from black or rnixed•race South Africans.

strains from Asians ($P < 10'$, Fisher's exact test) and the 58% of 40 strains from North Americans ($P < 0.005$). In contrast to this finding for the *vacA* signal region, both vacA mid-region types were observed among South African strains, as was the case for strains from Asia and North America. *cagA'* strains were observed in South Africa at a frequency similar to that for strains from the United States, whereas all the strains we have studied from Asia were $cagA^+$ (Fig. 1).

The only subject with a type s2 *vacA* signal region appeared to have an s2/ml *vacA* allele; natural occurrence of *vacA* alleles with this structure has not previously been reported. To confirm the genotype of this isolate, the 246-hp signal region and 463-bp mid-region PCR products from duplicate reactions were cloned into the pGEM-T Easy vector (Promega) and sequenced. The nucleotide and deduced polypeptide sequences were then compared with the corresponding regions of the published *vacA* sequences from United States strains 60190 (slalml) and Tx30a (s2/m2) by using the Clustal

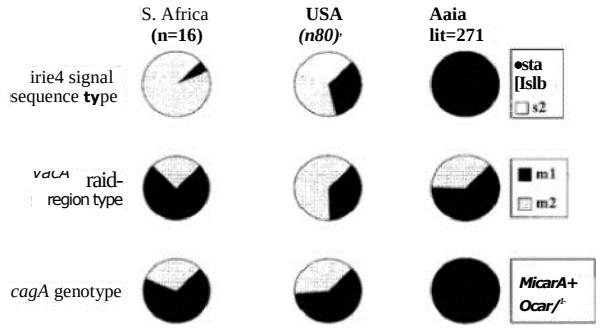


FIG. I. Comparison of v.:m.4 signal sequence and mid-region allelic types and *aigA* genotype between *H. pylori* isolates from South **Africa.** the United States (USA), arid Asia (Japan In = 131, China la = and Thailand In = 9)).

V algorithm (9). The signal region showed greatest identity (87.4% at the nucleotide level) with the typical United States s2 strain Tx30a, including the characteristic 9-codon insertion, which encodes a signal processing site different from that of the sl signal sequence (2). The mid-region showed greatest identity (99.1% at the nucleotide level) to the typical United States ml strain 60190.

For a subset of isolates (a maximum of four strains of each genotype), cytotoxin activity was determined by applying 48-h unconcentrated broth culture supernatants to cultured AGS cells, incubating the cells for 24 h at 37°C, and recording the level of vacuolation observed (2). Two of the strains were cytotoxic in this assay (defined as >80% of HcLa cells exhibiting vacuolation), the *vacA* slafml strain and one of the sib/ ml strains; thus, the 52./m1 strain was noncytotoxic (Table O.

To conclude, *vacA* diversity was demonstrated among South African *H. pylori* strains, hut the pattern was different from that found among strains from the United States or Asia. In particular, strains with the *vacA* sla genotype were not found among Hp *pylori* isolates from black or mixed-race South Africans in this sample, High levels of *H. pylori* infection exist in Africa, yet the incidences of peptic ulcer disease and gastric adenocarcinoma are both thought to he low (10). It is interesting to speculate that this may he explained by a low prevalence of *H. pylori* strains with the *vacA* sla allele. (Infection with strains with the *vacA* sla allele has been shown to be associated with peptic ulceration in a United States population [4].) A larger study to examine the association between *vacA* genotype, race, and disease in South Africa is currently in progress. Perhaps the most important discovery in this study was the natural existence of a strain with a *vacA* s2/m1 genotype. The finding that all combinations of *vacA* signal sequence and mid-region do occur naturally strongly supports the concept of recombination occurring between *vacA* genes in vivo to create the mosaic gene structures observed,

We thank Rachel Twells and Brian Dove for their technical assistance.

This work was funded by the Medical Research Council (United Kingdom) and the British Digestive Foundation.

REFERENCES

1. **Allierlith, .J. C.** 1997. Molecular methods for detecting ulcerogenic strains of N. pyFori, **p. 133-143.** ***hi*** C. L. Clayton arid H. L. T. Mobley (ed.), *liclicobacfcr pylori* protocols. Humana Press Inc., Totowa, N.J.
2. **Atherton, J. C.. P. Can. IL M. Peek, M. K. IL Teatiinuru. M. J. Maser, and T. L.** Cover. 1995. Mosaieism in vacuolating eytotoxin alleles of *llelicobacier pvlori;* association of specific *frrrA* types with cytotoxin production and peptic ulceration. J. Din]. Client. 270:17771-17777.
3. **Atherton, J. C., M. Karita,** Gimmdea.videntia,Ivi. **R. Morales, K. C. Ray, R.** 70. Peek, G. t. Perez-Perez. T. L. Craver, and **M. J.** Plaser, 19%. Diver-My in *vacA* mid-region hut nos in signal sequence type among *f lalicnbacter pylori* strains From Japan, China. Thailand and Peru, Gut 39;A73.
4. Atherton, **J. C., R. M.** Peek, Jr.. K. T. Thum, T. L Cover, and **M. J.** Maser. 1997. Clinical and pathological importance of heterogeneity in *PacA,* the vacuolating qtatoxin gene of *Helirobarier pyloli.* Gastroenterology I12:9299.
5. Censini, S., C. Lange, Z. Xiang, J. E. Crabtree, P. Ghlara, M. Borodovsky, ***IL*** **Ramiro% mad A. Caviled. 1996.** *trig,* a pathogenicity island of *Hclicobactrr ',god,* encodes type I-specific and disease-associated virulence factors. Proc. Natl. Acad. Set USA 93:14648-14653.
8. Cover, T. L., M. K. R. Tummuru, P. Cao, S. A. Thompson. and M. J. Maser. 1994. Divergence of genetic sequences for the vactiolaiing eytoioxin among *Helirobacrer pylori* **strains.** J. BM!. Chem. 269:10.5.(41-111573.
7. Go. **M. F., V. Kapur, D. Y. Graham, and J. M. Musser. 19%.** Population genetic analysis of *firlicobatirr pylori* by multilocus enzyme electrophoresis: extensive allelic diversity and recomhinationa I population structure. J. Bacterial. 178:3934-3978.
S. **Hazel'. S. L, IL H. Andrews, H. M. Mitchell, and G. Daskahipouloms. 1997.** ienetic relationship among isolates of *lielicobaarr pvloii:* evidence far the existence of *a Helicabacier pylori* species-complex. **FEMS** Microbiol. Lett. 1.511:27-32.
9. **Higgins, D. G., and P. M. Sharp. 1919. Fast and** sensitive multiple sequence

alignments on a microcomputer. CABIOS **5:**151–153.
10. **Holcombe, C.** 1992. *Helicobacter pylori*: the African enigma. Gut **33:**429–431.
11. **Letley, D. P., R. J. Twells, B. K. Dove, C. J. Hawkey, T. L. Cover, and J. C. Atherton.** 1998. Determinants of *Helicobacter pylori* vacuolating cytotoxin production analysed by construction of *vacA* hybrids and by site-directed mutagenesis. Gastroenterology **114:**A1020–A1021.
12. **Leunk, R. D., P. T. Johnson, B. C. David, W. G. Kraft, and D. R. Morgan.** 1988. Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. J. Med. Microbiol. **26:**93–99.
13. **Salaün, L., C. Audibert, G. Le Lay, C. Burucoa, J. Fauchère, and B. Picard.** 1998. Panmictic structure of *Helicobacter pylori* demonstrated by the comparative study of six genetic markers. FEMS Microbiol. Lett. **161:**231–239.
14. **Telford, J. L., P. Ghiara, M. Dell'Orco, M. Comanducci, D. Burroni, M. Bugnoli, M. F. Tecce, S. Censini, A. Covacci, Z. Xiang, E. Papini, C. Montecucco, L. Parente, and R. Rappuoli.** 1994. Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. J. Exp. Med. **179:**1653–1658.

CHAPTER 3

NATURAL DIVERSITY IN THE N TERMINUS OF THE MATURE VACUOLATING CYTOTOXIN OF *Helicobacter pylori* DETERMINES CYTOTOXIN ACTIVITY

Letley, D. P. and J. C. Atherton (2000)

Journal of Bacteriology **182**:3278-3280

JOURNAL OF BACTERIOLOGY, June 2000, p. 3278-3280 Vol, 182, No. 11
0021-91931001$04.00+0

Natural Diversity in the N Terminus of the Mature Vacuolating Cytotoxin of *lielicobacter pylori* Determines Cytotoxin Activity

D. P. LETLEY* **AND** J. C. ATHERTON

Division of Gastroenterology and Institute of Infections and Immunity, University of Nottingham, Nottingham NG7 2UH, United Kingdom

Received 26 October 1944/Accepted 7 March 21X10

Naturally occurring noneytotoydc *vacA* type s2 strains of *ffefirobacrer pylori* have a 12-residue extension to the vacuolating cytotoxin (VacA) compared with cytotoxic type sl strains. We show that adding the region encoding this extension to type sl *vacA* completely abolishes lac:notating cytotoxin activity but has no effect on VacA production.

Essentially all strains of the gastric pathogen *Helicobacter pylon* possess *vacA,* the gene encoding its vacuolating cytotoxin (4). However, *vacA* alleles vary between strains, and one important difference is that two versions of the region encoding the signal peptide commonly occur, termed si and s2 (1). This is medically important, because in the United States, *IL pylori* strains with type sl *vacA* are usually cytotoxic in vitro and are associated with high levels of gastric inflammation in vivo and a high prevalence of peptic ulceration (2). In contrast, strains with type s2 *vacA* are not cytotoxic in vitro, are associated with less inflammation in vivo, and are rarely isolated from patients with peptic ulcers (2). Why differences in the region encoding the VacA signal peptide have these associations is unclear, but it has been speculated that there may he differences in signal peptide cleavage efficiency or that the signal region differences may be a marker for more subtle sequence differences in *vacA,* either upstream (affecting level of transcription) or downstream (affecting VacA structure). In previous investigations, we have shown that the signal sequence cleavage site differs between sl and s2 VacA: mature type sl VacA has a hydrophobic N terminus, hut type s2 VacA has a short, predominantly hydrophilic, 12-amino-acid, N-terminal extension (1). This led us to hypothesize that the naturally occurring N-terminal extension of s2 forms of VacA may "block" toxin activity. We have been encouraged in this work by recent reports from de Bernard et al. and Ye et al., who have worked on the toxigenic sl form of VacA and who have shown that deleting the hydrophobic N terminus (9 and 17 amino acid residues, respectively) from sl VacA abolishes vacuolating activity when the modified toxin is expressed intracellularly (5, 7).

To investigate the importance of the s2-specific hydrophilic extension, we inserted the DNA encoding this region from the nontoxigcnic s2 strain Tx30a (ATCC 51932) into the corresponding position in *vacA in* the toxigenic sl strain 60190 (ATCC 49503). To do this, we first cloned the 5' 274 bp of vacA from 60190 into pBluescript SK together with the 3'-terminal region of the upstream gene cys.S. Next, we inserted a chloramphenicol resistance marker 74 hp upstream of the *vacA* promoter, and constructed a 60190 control strain by transforming 60190 with this construct. We then introduced the 36-hp insertion encoding the s2-specific 12-amino-acid extension (nucleotides 436 to 471 in the Tx30a *vacA* sequence, GenBank accession number U2940]) into the cloned *vacA* sequence immediately downstream of the region encoding the signal peptide (between cottons 33 and 34 in the 60190 *vacA* sequence, GenBank number U05676). We did this by inverse PCR using primers which annealed to 60190 *vacA adjacent* to the site of insertion (896 to 923 nucleotides and 868 to 895 nucleotides respectively). Each primer contained half of the sequence to be inserted at its 5' end. Following inverse PCR, we recircularized the linear product by blunt-end ligation and confirmed the presence of the correct 36 by insertion by nucleotide sequence analysis. We introduced the resulting construct into the chromosomal *vacA* gene of 60190 by allelic exchange, using natural transformation and marker rescue. We confirmed the presence of the insertion in 60190 *vacA* by PCR analysis using allelic type-specific primers (I). To confirm that the signal peptide cleavage site was not altered by the presence of the hydrophilic extension, we determined the N-terminal amino acid sequence of the mature, secreted VacA protein of the 60190 N-terminal insertion mutant strain (Applied Biosystems 473A, Warrington, United Kingdom). The N-terminal sequence, NTPNDP, confirmed the presence of the s2-specific hydrophilic extension.

Next, we assessed the vacuolating activity of the 60190 control and its isogenic N-terminal insertion mutant derivative, 60190 *vacA::N(s2),* by incubating HeLa, AGS (a human gastric adenocarcinoma cell line), and RK-13 (a rabbit kidney epithelial *cell* line) cells overnight with unconcentrated 48-h broth culture supernatants (grown in Oxoid Iso-Sensitest broth supplemented with 5% fetal calf serum). For all six control 60190 broth supernatants assayed, essentially all cells were vacua-lated fur each of the three cell lines, whereas incubation with the six supernatants from 60190 *vacA::N(s2)* produced no *vac*-uolation in any of the cell lines tested (Fig, 1). To confirm that loss of vacuolating activity was not due to a loss of VacA expression in our mutant strain, we performed immunoblots and enzyme-linked immunosorbent assays (ELISAs) for mature VacA protein in the same 48-h broth culture supernatants by using an antiserum raised against the recombinant midre-gion of VacA from strain 60190 (kindly donated by T. L. Cover, Vanderbilt University, Nashville, Tenn.). The immuno-blot showed that a protein of similar size (-87 kDa) and amount was detected in all six 60190 controls and all six 60190

■ **Corresponding author. Mailing address: Division of** Gastroenterology, Queen's Medical Centre, University Hospital, Clifton Blvd., Nottingham NG7 2UH, United Kingdom. Phone: (44) 115-9249924. Fax: (44) 115-9709923. E-mail: Darren.Lctley@ nottingham.ac.uk.

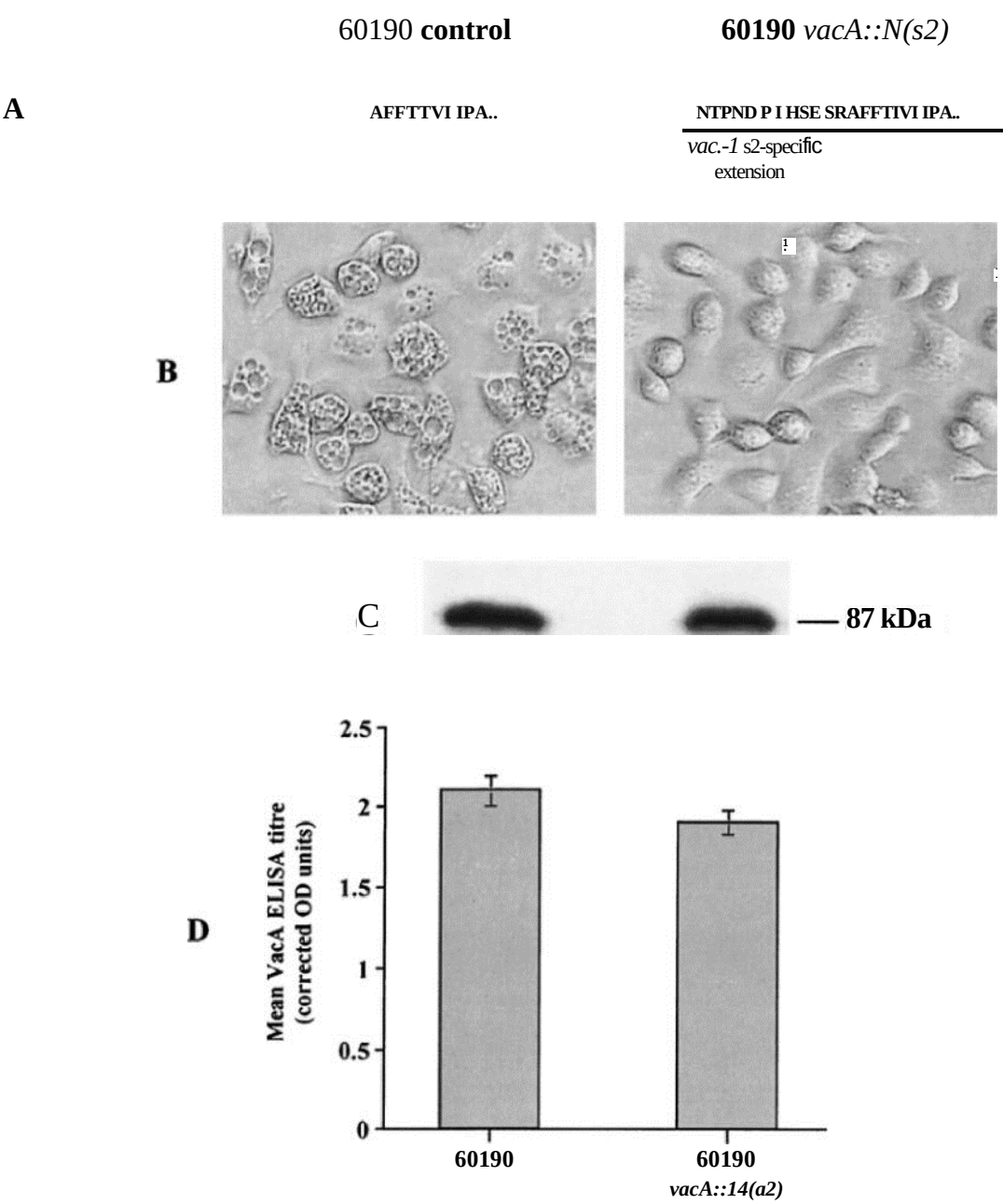


FIG. 1. Vacuolating activity and VacA production of the 60190 control strain and its N-terminal insertion mutant derivative, 60190 vacif::N(s2). (A) Difference in the N-terminal amino acid sequence of mature VacA between wild type 60190 and 60190 vac,4::N(s2). The presence of the s2-specific N-terminal extension in VacA from strain 60190 vac,·L:N(s2) was confirmed fur the first six residues by N-terminal peptide sequencing. (B) The effect on AGS cells following overnight incubation with 48-h broth culture supernatant of either the 60190 control strain (left) or 60190 *varill:N{s2)* (right) diluted fivefold in RPM] 1610 medium (Gibcci BRL) containing III niM ammonium chloride. The presence of cytoplasmic vacuolation was assessed visually by light microscopy at a x50 magnification. (C) Immunoblot of 48-h broth culture supernatant of 60190 control (left lane) and 60190 mcel::N(s2) (tight lane) detected with rabbit antisera raised against the recombinant VacA type ml midregion, (D) Quantification of VacA in etti-h broth culture supernatants by ELISA. Mean VacA ELISA titers, corrected for bacterial density, of six 60190 vocA:14(s2) and three 60190 control strain supernatants arc shown. OD, optical density_

vacA::N(s2) mutant broth supernatants tested, confirming that full-sized, mature VacA protein was consistently secreted by the mutant strain, ELISAs showed that similar levels of VacA were produced by 60190 *vac"* *(s2)* and by the 60190 control (means -± standard deviations were 1.9 -± 0.18 *[n* = 6J and 2.1 ± 0.16 *[n* = 3] corrected optical density units respectively, *P* = 0.15, *t* test).

In conclusion, we have shown that adding the hydrophilic, 12-amino-acid, N-terminal extension, which is specific to VacA type s2 strains, to the N terminus of type sl VacA abolishes

vacuolating cytotoxin activity. Loss of vacuolating activity is not explained by differences in VacA production or stability, because both are unaffected by the addition of the s2-specific region. Our results raise the interesting question of why *vacA* s2 strains of *H. pylori* (producing the blocked s2 form of toxin) survive and thrive in apparent competition with *vacA* s1 strains. This is particularly curious given the freely recombinational population structure of *H. pylori* (6), which should amplify any negative selection pressure. This and the fact that sequence homology among type s2 *vacA* alleles is as close as among type s1 alleles (3) lead us to speculate that the "blocked" s2 form of VacA has an important function for the bacterium, and we are investigating what this function may be. Our results are also of major medical importance, for they explain why *H. pylori* strains with the s2 form of *vacA* are nontoxigenic, cause little inflammation, and are rarely associated with peptic ulceration. These results also provide a rational basis for testing for *vacA* signal region type.

We acknowledge the technical assistance of Rachel Twells, Brian Dove, and Joanne Bower.

This work was funded by the Medical Research Council (United Kingdom).

REFERENCES

1. **Atherton, J. C., P. Cao, R. M. Peek, M. K. R. Tummuru, M. J. Blaser, and T. L. Cover.** 1995. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*: association of specific *vacA* types with cytotoxin production and peptic ulceration. J. Biol. Chem. **270:**17771–17777.
2. **Atherton, J. C., R. M. Peek, Jr., K. T. Tham, T. L. Cover, and M. J. Blaser.** 1997. Clinical and pathological importance of heterogeneity in *vacA*, the vacuolating cytotoxin gene of *Helicobacter pylori*. Gastroenterology **112:** 92–99.
3. **Atherton, J. C., P. M. Sharp, T. L. Cover, G. Gonzalez-Valencia, R. M. Peek, S. A. Thompson, C. J. Hawkey, and M. J. Blaser.** 1999. Vacuolating cytotoxin (*vacA*) alleles of *Helicobacter pylori* comprise two geographically widespread types, m1 and m2, and have evolved through limited recombination. Curr. Microbiol. **39:**211–218.
4. **Cover, T. L., M. K. R. Tummuru, P. Cao, S. A. Thompson, and M. J. Blaser.** 1994. Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. J. Biol. Chem. **269:**10566–10573.
5. **de Bernard, M., D. Burroni, E. Papini, R. Rappuoli, J. Telford, and C. Montecucco.** 1998. Identification of the *Helicobacter pylori* VacA toxin domain active in the cell cytosol. Infect. Immun. **66:**6014–6016.
6. **Suerbaum, S., J. Maynard-Smith, K. Bapumia, G. Morelli, N. H. Smith, E. Kunstmann, I. Dyrek, and M. Achtman.** 1998. Free recombination within *Helicobacter pylori*. Proc. Natl. Acad. Sci. USA **95:**12619–12624.
7. **Ye, D., D. C. Willhite, and S. R. Blanke.** 1999. Identification of the minimal intracellular vacuolating domain of the *Helicobacter pylori* vacuolating toxin. J. Biol. Chem. **274:**9277–9282.

CHAPTER 4

DETERMINANTS OF NONTOXICITY IN THE GASTRIC PATHOGEN *Helicobacter pylori*

Letley, D. P., J. L. Rhead, R. J. Twells, B. Dove and J. C. Atherton (2003)

Journal of Biological Chemistry **278**:26734-26741

THE JoirENAI,CR BunnrTrxi. Cacalecrer
2003 by The American Society for Biochemistry and Molecular Biology, Inc

Vol. 278. No. 29, Issue of July 15, pp. 26734-28741, 2003
Printed in *USA.*

Determinants of Non-toxicity in the Gastric Pathogen *Helicobacter pylori**

Received for publication, April 17, 2003
Published, JEC Papers in Press, May 8, 2003, DOI 10.1074/jbc.M304071200

Darren P. Letleyt, Joanne L. Rhead, Rachel J. Twells, Brian Dove, and John C. Atherton*

From the Division of Gastroenterology and Institute of infections, Immunity and Inflammation, University of Nottingham, C Floor, West Block, Queen's Medical Centre, Clifton Boulevard, Nottingham N07 2UH, United Kingdom

The *Helicobacter pylori* vacuolating cytotoxin gene, vacA, is naturally polymorphic, the two most diverse regions being the signal region (which can be type sl or s2) and the mid region (ml or m2). Previous work has shown which features of *vacA make* peptic ulcer and gastric cancer-associated type slim' and s1/m2 strains toxic. vacA s2/m2 strains are associated with lower peptic ulcer and gastric cancer risk and are non-toxic. We now define the features of vacA that determine the non-toxicity of these strains. To do this, we deleted parts of *vacA* and constructed isogenic hybrid strains in which regions of vacA were exchanged between toxigenic and non-toxigenic strains. We showed that a naturally occurring 12-amino acid hydrophilic N-terminal extension found on s2 VacA blocks vacuolating activity as its removal (to make the strain sl-like) confers activity. The mid region of s2/m2 *vacA* does not cause the non-vacuokiting phenotype, but if VacA is unblocked, it confers cell line specificity of vacuolation as in natural s1/m2 strains. Chromosomal replacement of *vacA* in a non-toxigenic strain with vacA from a toxigenie strain confers full vacuolating activity proving that this activity is entirely controlled by elements within vacA. This work defines *why H. pylori* strains with different vacA allelic structures have differing toxicity and provides a rational basis for *vacA* typing schemes.

Gastric colonization by *Helicobacter pylori* is the main cause of peptic ulceration, gastric carcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (1-3). However, although more than half the world's population is chronically infected with this Gram-negative bacterium, most people remain asymptomatic. Who develops disease depends on strain virulence, host genetic susceptibility, and environmental factors. Several bacterial virulence factors have been linked with disease: the active form of the vacualating cytotoxin, VacA (4, 5); presence of a pathogenicity island encoding a type IV secretion system, *cog* (6-8); and possession of a specific adhesin, BahA (9),

The active form of VacA induces extensive cytoplasmic vacuolation in epithelial cells (10), causes gastro-duodenal damage in a mouse model (11), and increases gastric ulcer risk in ***H.*** pylori-infected Mongolian gerbils (12). The vacA gene encodes a preprotoxin of -139 kDa (13-16). This includes an N-terminal signal peptide and a -50-kDa C-terminal autotransporter domain, both of which are cleaved during toxin secretion through the bacterial membranes (15). The -90-kDa mature toxin may undergo further processing at an exposed protease-sensitive loop into -37-kDa N- and --58-kDa C-terminal fragments (p37 and p58, respectively) (15, 17), The toxin binds to cell surface receptors through the p58 domain (18-20). However, when expressed in epithelial cells, only p37 and an N-terminal fragment of p58 are required for vacuolation (21). Vacuolation is dependent on the insertion of VacA multimers into cell membranes to form anion selective pores (22, 23). The mechanism of subsequent vacuole formation and the cellular origin of the vacuoles from late endosomes have been studied extensively (24-30).

Research has focused on the most active form of VacA, but most *H. pylori clinical* isolates express less vacuolating or non-vacuolating forms (31). The gene, *vacA,* is naturally polymorphic and differences are most marked in two areas: the signal region, encoding the signal peptide and the N terminus of the mature protein (which may be type sl or s2) and the mid region, encoding part of the p58 domain (type ml or m2) (31). *vacA* signal and mid regions from **all** clinical isolates of *H. pylori can* be classified as one of these types, and all combinations of *signal* and mid region occur naturally, although the s2/m1 structure is rare (31, 32). The *vacA* allelic type is associated with vacuolating activity *in vitro:* strains with slim' *uacA* cause more extensive vacuolation in HeLa cells than those with slim2 *vacA,* and *melt* s21m2 strains are invariably non-vacuolating (31). The existence of stable polymorphisms affecting toxin function is of major biological interest; it may form a paradigm for varying functionality of proteins in other bacteria with high levels of genetic recombination in which mosaic genes are common. However, for ***H. pylori, vacA*** polymorphism is also potentially of major clinical importance: *vacA* s21m2 strains are less frequently associated with both peptic ulceration and gastric carcinoma than *vacA al rat* or sl/m2 strains (33-38).

In this report, we define the determinants of non-toxicity in *H. pylori* with type s2/m2 *vacA.* Work by us and others on the most toxic slim' type of *oath* has guided our approach. We have previously shown that *vacA* transcription is higher for some toxic than for some non-toxic strains (39). We and others have also shown that the N terminus of mature sl/ml VacA in toxic strains is one important determinant of toxicity and that adding an s2-like N-terminal extension blocks activity (21, 40-43). However, one cannot extrapolate from this to imply that the N terminus of natural s2im2 strains is the cause of their non-toxicity, **so we** aimed to determine whether this was indeed the case. Naturally occurring *vacA* type slim' strains *cause* vatualatien in a wider range of cell lines than slirn2 strains (19, 44), so we also planned to show whether the m2 mid region was the cause of non-toxicity in natural s2/m2 strains.

This work was supported in part by a grant from the Medical Research Council (MRC) (United Kingdom). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore he hereby marked *"advertisement"* in accordance with *18* U.S.C. Section 1734 solely to indicate this fact.

To whom correspondence should be addressed. Tel.: 44-0-115-970-9063; Fax: 44-0-115-970-9923; E-mail:darren.letley@nottingham.ae.uk. § Supported by an MRC Senior Clinical Fellowship.

TABLE I
H. pylori strains and plasmids

Strain/plasmic'	Relevant characteristics	Source
[11]- pylori Tx30a		
60190 84-183	Wild type (ATCC 51932); *tawA* s2/m2; *cag*-	Ref, 10
Tx30a/CAT	Wild-type (ATCC 49503); vacA sl./m1; *cog*'	Ref. 10
Tx30a/P1S1	Wild-type (ATCC 53726); *vacA* sl/ml; *cog*	Ref. 64
Tx30a/N1	As Tx30a except *cat* inserted immediately 3' to *cysS;* **C111**[11]	This study
Tx30aIKAN	**As** Tx30a/CAT except *uacA* promoter/signal region replaced with sl type to nt 763	This study
Tx30a/M1	As Tx30aICAT except in-frame deletion of ircecA nt 436-471	This study
Tx30a/P1S1M	Ac Tx30a except *aphA* inserted into *fecE; Kink*	This study
1	As Tx30a/KAN except 3' *vacA replaced with ml* type from nt 843	This study
60190/CAT	*As* Tx30a/KAN except *vocA* promoter/signal region replaced with sl type to nt 638; cat inserted immediately 3' to *cysS; Cm"*	This study
60190/P2S1	As 60190 except *cat* inserted immediately 3' to *cysS;* Cm"	This study
60190/P2S2	As 60190/CAT except vacA promoter region replaced with s2 type to nt 795	This study
60190/RAN	As 60190/CAT except *vacA* promoter/signal region replaced with e2 type to nt 1070	This study
60190/M2 84-183/P2S2	As 60190 except aphA inserted into *fecE;KmR*	This study
	As 60190/KAN except 3' *vacA* replaced with m2 type from nt 1710	This study
84-163/P151,,,,,1.	As 84-183 except *uacA* signal and promoter region replaced with s2 type; *cat* inserted immediately 3' to *cysS;* Cm"	This study
	As 84-183 except *vacA* signal and promoter region replaced with el type from 60190; cat inserted immediately 3' to cysS;	This study
Plasmids		
pA153		
pA163::cat	pBluescript containing 3' *cysS* to vacA nt 418 from strain Tx30a; Amp' *As*	Ref. 31
pCTB2	pA153 except *cat* inserted immediately 3' to *cysS;* Amp"; Cm'	This study
pCTB2:Trot	pBluescript (Stratagene) containing 3' *cysS* to vocA nt 274 from strain 60190; Amp'	Ref. 13
pNV2	As pCTB2 except cat inserted at HindlII site immediately 3' to *cysS:* Amp"; Cm"	This study
	pBluescript containing a 5.2-kb fragment from 60190 including the 3' 3587 nt of vocA, complete *lecE,* and partial *,fecD* sequences, *fecE is* interrupted by *aphA*	This study
pNV5	located at XmnI *site;* Amp"; KmR	
	pBluescript containing a 4.7-kb fragment from Tx30a including the 3' 3676 nt of *vacA,* complete *fecE,* and partial *fecD* sequences, *fecE is* interrupted by *aphA* located at its single *Xmnl site;* Amp"; Km"	This study

Finally, it is unclear whether elements outside vacA in s2/m2 strains contribute to non-toxicity, and we aimed to resolve this issue,

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and *Gmwth Conditions—H. pylori strains* (Table I) were grown on blood agar base 2 supplemented with 7% (v/v) horse blood (Oxoid Ltd.) microaembically at 37 °C (Campypak Plus, BD Biosciences UK Ltd.). Broth cultures were grown in Iso-sensitest media (Oxoid Ltd.) with 5% (v/v) FCS' (Invitrogen) microaero-bically with shaking.

Construction of Isogenk vacA *Signal Region Hybrid Strains—The* effect of *uacA* signal region type on toxin production and activity was studied by constructing *H. pylori* isogenic *uacA* hybrid strains in which different extents of the signal region had been exchanged between the sl and s2 forms on the *ft pylori* chromosome. Such hybrids were constructed using plasmids pA153::cat and pCTB2::cat, containing the promoter and 5' terminus of *vacA,* including the signal region, from strains Tx30a *(vacA* s2/m2; Tox– } and 60190 *(vacA* 81/m1; Tox+), respectively, together with the 3' terminus of the upstream gene *cysS with* a chloramphenicol resistance marker (chloramphenicol acetyl-transferase; *cat)* inserted immediately downstream (see Table D. These pBluescript-derived plasmids act as suicide vectors in *H. pylori,* thus chromosomal recombinants were constructed by natural transformation, allelic exchange, and chloramphenicol rescue as previously described *(41) (see* Fig. 1). Transformation of pA153::cat and pCTB2::cat into their respective homologous strains resulted in the isolation of control strains Tx30a1CAT and 60190/CAT, containing the insertion of *cat* upstream of the *vacA* promoter. Transformation of Tx30a with pCT.82.::cat and 60190 with pA153:iroi gave rise to multiple hybrid strains. DNA extracted from single colonies (45) was typed for *uttrA* signal and mid region by allele-specific PCR, as previously described (31, 46). Only vacA type al recombinants were identified from the transformation of Tx30a with pC'T132::cai, and one was selected and termed Tx30a/P1S1. For the transformation *of* 60190 with pA153::cat, type sl and s2 recombinants were identified and termed 60190/P2S1 and 60190/P252, respectively. The precise extent of recombination was determined for these mutants by nucleotide sequencing. pA153::cat and peTB2rIcat were similarly transformed into the toxigenic strain $4-163 (vacA sl/ml) to *give* hybrids 84-183/P2S2 and 84-183/1[2]1S1,,,,,, respectively.

Construction of vacA Mid Region Hybrid Strains—The effect of *vacA* mid region type **was** studied ins *similar* manner. Plasmids pNV5 and pNV2, containing 3' terminal 3576 bp and 3587 bp of *vacA* with 1 kb and 1.5 kb of downstream sequence from strains Tic30a and 60190, respectively, with a kanamycin resistance marker (aminoglycoside phosphotransferase; *aphA)* inserted 0.5 kb 3' to vacA, within the *fecE* gene, were introduced into strain Tx30a by natural transformation, allelic exchange, and kanamycin marker rescue. Recombinants were typed for vacA signal and mid region by allele-specific PCR (31, 46). Transformation with pNV5 and pNV2 gave rise to the control strain Tx30a/KAN and the hybrid strain Tx30a/M1 (vacA s21m IL respectively. Similarly, transformation of strain 60190 with pNV2 and pNV5 created the control strain 60190/EAN and the hybrid strain 60190/M2 (vacA s11m2), respectively. Tx30a/M1 was further transformed with pCTB2-:cat to produce the hybrid strain Tx30a/P151M1 *(vacA 811m1).*

Site-directed Mutagencsis of vacA—The *vacA* mutant Tx30a/N1, containing an in-frame deletion of the region encoding the s2-specific N-terminal extension, was constructed by first deleting this 36-bp region from the cloned *uacA* fragment in plasmid pA153::cat using inverse PCR with primers NdelF rt. fCACAACCG'TGATCATTCCA-GCC-3') and NdelR (5'-AGCCCCCAOTFCGOTGCCCA'TTAACACCC-3'), which bound to nucleotides 472-501 and 107-435 in the *vacA* sequence of Tx30a (3I), respectively, Template DNA was removed by DpnI restriction endonuclease digestion (New England BioLabs (UK) Ltd.), and the PCR product was end-polished using *Pfu* DNA polymerise (Stratagene Europe), 5'-phosphorylated with T4 polynucleotide ki-nase in the presence of 1 mat ATP, and blunt-end-religated. Following transformation into *Escherichio cult* strain Dff5a, plasmid DNA was extracted, and the presence of the 36-bp deletion screened by PCR using primers VA1F and VA1R (31), and confirmed by nucleotide sequencing. The *vacA* mutation was introduced into chromosomal uacA of *H. pylori* strain Tx30a by natural transformation, allelic exchange, and chloram-phenicol marker rescue, and the presence of the vacA mutation was confirmed by PCR as before.

***Quantification** of VacA—VacA* production was quantified by antigen detection ELISA as previously described (47). Briefly, duplicate 48-h broth culture supernatant samples of each strain were adsorbed to a microti ter plate overnight at 4 °C, blocked with 3% bovine serum albu-

' The abbreviations used are: FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltranaferase.

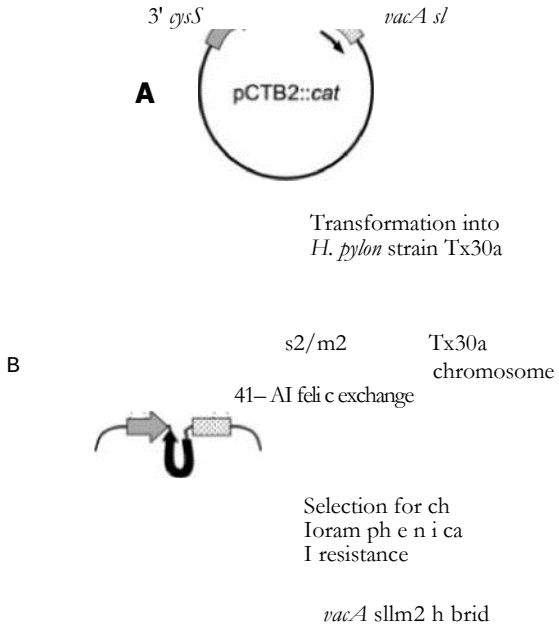
min (Sigma), and bound antigen incubated with a 1:5,000 dilution of rabbit antiserum to either purified VacA from the type sliml strain 60190 (Ab123) (47), or the recombinant VacA p58 fragment from either this strain (Ab929) (18) or the m2 type strain Tx30a (Ab927), all kindly donated by Dr. T. L. Cover, Nashville, TN, For detection we used anti-rabbit IgG-horseradish peroxidase conjugate (Sigma) then ortho-phertyldiamine/hydrogen peroxide. ELISA values were expressed as .i1 45 h., corrected for bacterial density (A600.,,,,).

Detection of VacA by Immunoblotting—H. pylori broth culture supernatants or water extracts were separated by SDS-PAGE and transferred to nitrocellulose by eiectroblotting. Nitrocellulose blots were blocked in 5% (w/v) milk in PBS-Tween 20 (PBS-T) then incubated with rabbit anti-VacA antiserum (Ab123, 927, or 929) diluted 1:10,000. Blots were washed three times in PBS-T, incubated in anti-rabbit IgG-horse-radish peroxidase conjugate, washed six times in PBS-T, and visualized by ECL^{rrm} detection (Amersham Biosciences).

Determination of VacA Actieity—Water extracts were prepared by harvesting 48-h growth from a single plate into 1 ml of sterile distilled water, vortexing for 30 s, and incubating at room temperature for 20 min. Cells were removed by microcentrifugation, and the supernatant containing VacA was filter-sterilized. Vacuolating activity was determined using three different epithelial cell lines: HeLa, AGS (a human gastric adenocarcinoma cell line), and it1{13 (a rabbit kidney cell line). Assays were performed by adhering 10^4 epithelial cells in 1tPM1 1640 media supplemented with 10% FCS (both Invitrogen) to a microtiter plate overnight. The media via, than replaced with fresh media containing 10 mm :iron-lin-141m chloride, anti a 5-fold dilution of water extract. Cells were incubated overnight and then visually assessed for vacuolation by light microscopy.

RESULTS

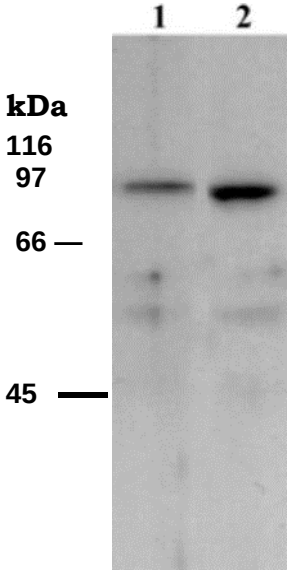
The Type s2 Promoter/ Signal Region of vacA Determines the Non-toxic Status of the Tox– Strain Tx30a—Our previous work had shown that *vacA* siirril strains are usually toxic and *vacA* 82/m2 strains invariably non-toxic (31). Our first aim was to define whether the promoter/signal region of a candidate nontoxic *vacA* s21m2 strain, Tx30a, was directly responsible for its non-vacuolating phenotype. To do this, we used allelic exchange to replace the promoter and signal region of vacA on the Tx30a chromosome with that of strain 60190 (tox+; vacA all ml) to make an artificial hybrid al/m2 *vacA* construct in a non-toxigenic strain background, which we called Tx30a/P181 *(see* "Materials and Methods" and Fig. 1). Nucleotide sequencing of this hybrid showed the crossover point to be between 588 and 763 in the Tx30a sequence (31) and proved that the promoter region was identical to that in the sl/ml strain 60190. To ensure that insertion of the chloramphenicol resistance marker *(cat),* used for the allelic exchange experiment, was not influencing VacA production or activity, we also constructed the control strain Tx30a/CAT with *cat* inserted between *cysS* and *vacA* (see "Materials and Methods"). Insertion of the chloram-phenicol cassette in Tx30a did not affect Vac-A. levels in broth culture supernatants, as determined by antigen detection EL1SA, and all later comparisons used Tx30a/CAT as control. Replacement of the signal and promoter regions with those from 60190 increased VacA production as determined by ELISA using antiserum Ab927 (Tx30a/CAT mean VacA production 0.03 ± 0.01, n = 10, *versus* Tx30a/P1S1 mean 0.17 0.05, n = 8, $p <$ 0.005, *t* test). This finding was confirmed by immunoblot using the same antiserum (Fig. 2). As expected, water extracts of the non-toxigenic control strain, Tx30a/CAT, did not cause vacuolation of any of the three cell lines tested (Fig. 3A and Table II). However, Tx30a/P1S1 water extracts induced extensive cytoplasmic vacuolation of RK13 cells following overnight incubation *(Fig.* 3B). Interestingly, the vacuolat-ing activity observed for this strain was cell line-specific, because the same water extracts were unable to induce vacuolation of HeLa and AGS cells (Table H). This is consistent with the described phenotype of a naturally occurring *vacA* sl/m2 strain (19). Thus, naturally occurring differences in *vacA*



Fie. 1. Construction of Tx30a/P1S1 **hybrid strain.** *A, a* fragment containing the 3' terminus of cysS, the *cysS-vacA* intergenie region and the first 274 nt of *vorA* from H. *pylori* strain 60190 (eacA sl/m1; Tox+) was cloned into pBluescript SE. A chloramphenicol acetyltransferase gene *(cat),* conferring resistance to chloramphenicol, was inserted **immediately** 3' to the *cysS* stop codon to create the plasmid pC7B2::cal. This plasmid was then introduced into the Tox– strain Tx30a *(vacA* s21m2) by natural transformation. ***B,*** in some transformed bacteria, DNA exchange occurs between the cloned *cysS-vacA* fragment and the equivalent chromosome sequence through homologous recombination, *C,* such recombination events were selected by growth on blood agar containing 30 ggfral chloramphenicol (pCT132::co t is unable to replicate autonomously in H, *pylori). Recombinants* were then *screened* by allele-specific PCR for the hybrid *vacA* type s1/m2,

signal regions are directly responsible for VacA production and activity_

Confirmation That the s2 Promoter / Signal Region of vacA Determines Non-toxic Status Using Tax+ Strain Back-grounds—Using identical methodology, we confirmed our results by performing reciprocal experiments in which we replaced the signal and promoter regions of *vacA* in strain 60190 (tox+ ; *vacA* sl/m1) with those of Tx30a (fox–; *vacA* s2/m2) to create the hybrid strain 60190/P2S2. Nucleotide sequencing confirmed that the *sequence* up to nucleotide 1070 had been replaced with that of Tx30a. As for Tx30a, we constructed a control strain of 60190 with the chloramphenicol cassette inserted just after the *cysS* stop codon; this did not affect VacA production or activity, and 60190/CAT was used as the control in all further experiments. Insertion of the signal and promoter region from Tx30a reduced VacA production as determined by ELISA using Ab123 (60190/CAT mean VacA production 1.01 0.12, n = 8, *versus* 60190/P252 mean 0.19 ± 0.02, n = 4, $p < 10'$). As expected, 60190/CAT water extracts caused extensive cytoplasmic vacuolation of all three cell lines studied (Fig. 4A and Table II). In contrast, no vacuolation of HeLa, AGS, or RK13 cells was observed with water extracts of the hybrid strain 60190/P252 (Fig, 4C and Table II). Next, we aimed to confirm our results in a third independent strain background. We selected strain 84-183, an easily naturally transformable strain of oacA type sliml, which we have previously shown to transcribe VacA less strongly than strain 60190 and to be less strongly vacuolating (39). Replacement of the signal and pro-



Fin. 2. Production of VacA by Tx30a control and hybrid strains. Water extracts of Tx30a/CAT *(lane I)* **and Tx30a/P1S1** *(lane 2)* **were separated by SDS-PAGE, transferred to nitrocellulose by electro-blotting for 1 h at 140 mA, and detected using a 1:10,000 dilution of antiserum to the recombinant p58 subunit of Tx30a VacA (Ab927). An immunoreactive band of --93 kDa was detected for the control strain Tx30a/CAT as reported previously (31). A more intense, but slightly smaller band was detected for Tx30a/P1S1 confirming that VacA production is increased in this strain. The difference in size is in agreement with the removal of the VacA N-terminal extension resulting from**
swapping the s2 signal region for the s1 type.

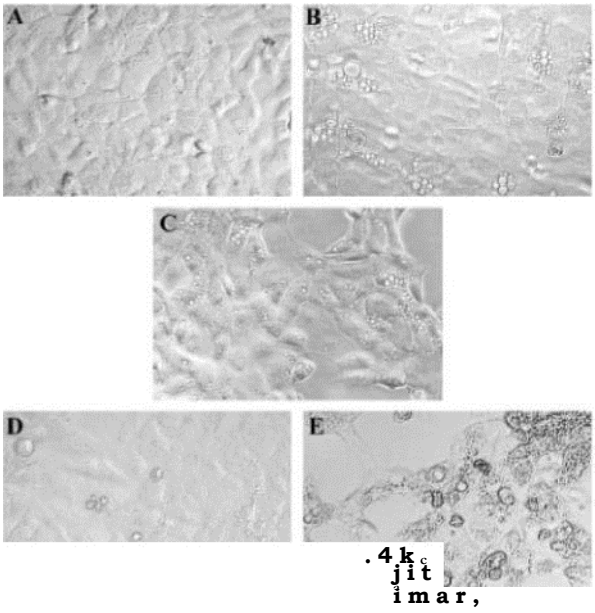


FIG. 3. Effect of Tx30a control and isogenic mutant water extracts **on** RK13 cells. **RK13 cells** were grown **in 96-well microtiter plates for 24** h (10^4 **cells/well) and then incubated overnight with 1/5 dilution of water extract of either strain Tx34a1CAT Tx30a/P1S1** *(B),* **Tx30a/N1 (C), Tx30a/M1** ***(D),*** **or Tx30a/P1S1M1** *(E).* Original magnification **was x50.**

moter regions of *oaeA* in strain 84-183 with those of strain Tx30a (to make strain 84-183/P2S2) reduced VacA production as determined by ELISA using Ab123 (84-183 mean VacA production 0.20 ± 0.02, *versus* 84-1831P2S2 0.05 ± 0.01, n = 6

TABLE II
Vacualating utotoxin activity of control and isagenic vacA hybrid ***H.*** *pylori strains*

Strain	*vacA* type	Vacuolating activity[1]		
		HeLa	MIS	RK13
Tx30a	**62/m2**			
Tx30a/CAT	**s2/m2**			
Tx30a/P1S1	**sl/m2**			
Tx30a/N1	s2s **1'im2**			
Tx30a/KAN	**s2/m2**			
Tx30a/M1	**82./m1**			
Tx30a/P1S1M1	**sl/ml**			
60190	**sl/m1**			
60190/CAT	**s1/m1**			
60190/P2S1	sllml			
60190/P2S2	**62/m1**			
60190/KAN				
60190/M2	**s 1/m2**			

Vacuolating activity was assessed by incubating either HeLa, AGS, **or RK13 epithelial cells overnight with a water extract of the appropriate strain. Vacuolation was recorded as positive if** more than 50% of the cells within a randomly chosen field were vacuolated and negative if the **number of vacuolated cells was the same or Less than that observed for untreated cells.**

[6] The signal region of this strain is s2 type for the **signal** peptide **including the cleavage site, and sl type for the mature N terminus.**

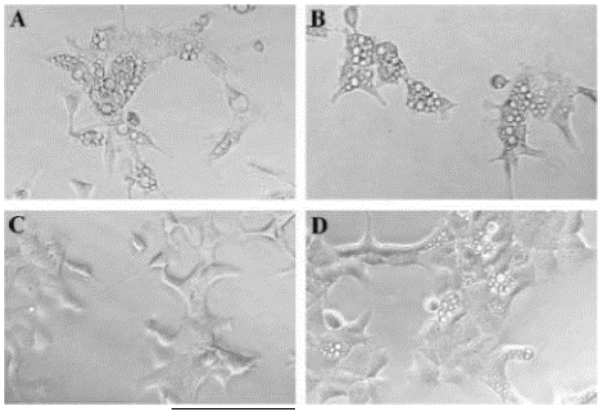
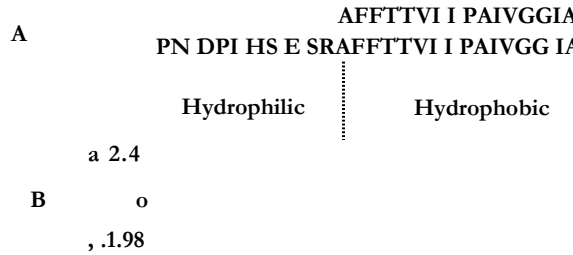


FIG. 4. Effect of 60190 **control and isogenic mutant water ex. tracts on RK13 cells. RK13 cells** were grown in **96-well microtiter plates for 24 h (10^1 celldwell) and then incubated overnight with 115 dilution of water extract of either strain 60190/CAT** ***(AL*** **60190/P2S1 (B), 60190/P2S2** *(C),* or **60190/M2 (DI. Original magnification was x50.**

for both, $p < 10^{-3}$). As expected, strain 84-183 caused vacuolation in HeLa cells, whereas strain 84-183/P2S2 did not (data not shown). In complementary experiments, we replaced the signal and promoter *regions* of strain 84-183 with those of strain 60190 (to make strain 84-183/P1S1$_{60190}$), and this increased VacA production (to mean 0.31 ± 0.02, n = 8, $p <$ 0.006). Both strains vacuolated HeLa cells (data not shown). These experiments show that replacing the type al promoter and signal region in a tox+ strain with a type s2 region from a tox – strain reduced VacA production and abolished activity. Interestingly, strains 60190/P2S2 and 84-183/P2S2, which both have a hybrid s2/m1 *meet* structure, grew similarly to their respective controls on blood agar and in broth. Thus the reason that strains of vacA type s2fml are uncommon in nature is not that they have obvious self-toxicity or a growth disadvantage, at least *in vitro.*

The vacA Promoter Region Determines Differences in VacA Production, but the Signal Region Determines Differences in Vacuolating Activity—Having shown directly that the *cocA* signal and promoter regions in vacA s2/m2 strain Tx30a were together an important determinant of its non-toxigenic status, we now aimed to define the role of each region individually.



Fin. 5. The N•terminal protein sequence of mature Vac& A, the N terminus of mature VacA from the s2-type strain Tx30a contains a 12-amino acid extension compared with that of the sl-type strain 60190. *B, a* mean hydrophobicity plot shows that in contrast to the hydrophobic mature N terminus of 60190 VacA, the N-terminal extension found on Tx30a VacA is hydrophilic.

From the previous experiments it was unclear whether the low level of VacA production in strains Tx30a and 60190/P2S2 was the reason these strains were non-toxic or whether there were other determinants of non-toxicity. We aimed to address this by screening for transformants from our previous experiments where the recombination point was between the promoter region and signal sequence coding region. Screening of mutants generated by transforming strain Tx30a with the promoter and signal regions of *vacA* from 60190 yielded no such transformanta. However, the transformation of 60190 with pA153::cat yielded a transformant, which typed as s1 by allele-specific PCR, and we called this 60190/P2S1. Sequence analysis showed that this hybrid contained a promoter region and ribosomal binding site identical to that of the type s2 strain Tx30a, but the signal sequence remained 60190 *vacA.* The exact 3' crossover point was between nucleotides 795 and 835 in the 60190 vacA sequence (13). Studying this hybrid allowed us to determine the relative effects of the s2 promoter and signal regions in the 60190 strain background. Replacing the promoter region only, significantly reduced VacA ELISA levels using Ab123 (60190/P2S1 mean 0.34 -1 0.09, n = 4, $p < 0.005$, *versus* 60190/CAT, not different from 60190/P2S2). However, in contrast to 60190/P2S2, which did not express vacuolating activity, 601901P2S1 *caused* vacuolation in all three cell lines (Fig. 4B and Table II). Thus the lack of vacuolating activity observed for 60190/P2S2 was not simply due to reduced VacA production as 601901P2S1 produced similar levels of VacA but still retained vacuolating activity. This shows that, as expected, the promoter region, not the signal region, determines VacA production. However, the signal region is responsible for determining VacA activity.

The N-terminal Hydrophilic Extension on Mature Type s2 VacA Determines Its Non-vacuolating Phenotype—A striking difference in the signal region between vacA type s 1 and s2 strains is the presence of a 12-amino acid extension to the non-toxigenic, s2 form of VacA (31). The mature N terminus of sl type VacA is markedly hydrophobic, whereas the 52-specific, N-terminal amino acid extension is strongly hydrophilic (Fig. 5). We hypothesized that the presence of this hydrophilic extension blocks the vacuolating activity of VacA produced by s2 strains such as Tx30a. To test this, we made an in-frame deletion in Tx30a vacit of the 36 nucleotides encoding this extension, thus removing it from the mature VacA product (see "Materials and Methods"). We named the resulting isogenic mutant strain Tx30a/N1. Removal of the N-terminal amino acid extension reversed the non-vacuolating phenotype of Tx30a, such that water extracts of Mc30a/N1 caused extensive vacuolation of RK13 cells (Fig. 3C). Tx30a/N1 did not vacuolate AGS or HeLa cells (Table II). Note that Tx30/N1 has a type m2 mid region, so this result is consistent with the described phenotype of a naturally occurring vacA sl/m2 strain (19). To ensure that the vacuolating activity of Tx30a/N1 was not due to a greater VacA production compared with Tx30a/CAT, we determined the amount of VacA in water extracts by ELISA using Ab123. VacA levels were no greater for Tx30a/N1 than Tx30a/CAT (means 0.11 0.02 for both, n = 10 and 4, respectively, p – ns). Thus, the non-toxigenic phenotype of the vacA s2/m2 strain Tx30a is due to the hydrophilic extension on the N terminus of mature VacA.

Replacing the m2 Mid Region of a Non-toxigenic Strain with an ml Mid Region Is Not Sufficient to Render It *Toxigenic*—Having shown that non-toxic vacA s2/m2 strains could be rendered toxic by replacing the signal region with an *al* region or by removing the N-terminal amino acid extension on the mature VacA protein, we now aimed to assess whether these strains could also be rendered toxic by changing the mid region to an ml type. To do this we replaced the *vacA* mid region of strain Tx30a with that of strain 60190 (tox+; *vacA sltml)* to make a *vacA* 32/m1 construct in a non-toxigenic strain background, which we termed Tx30a/M1 (see "Materials and Methods"), Sequence analysis confirmed that the vacA mid region sequence had been exchanged for the 60190 sequence downstream of nucleotide 828. This strain also gained the 3' region of vacA from 60190, but this region, which encodes the C-terminal bacterial outer membrane transporter, which is cleaved from mature VacA after export, is very similar between all *vacA* alleles (48). As the kanamycin resistance marker used for allelic exchange was located 3' to vacA within *fecE,* we also constructed the control strain Tx30a/KAN containing just the marker insertion.

Insertion of the kanamycin cassette in *fecE* did not affect growth on blood agar or VacA activity (Table II), and all later comparisons used Tx30a/KAN as the control. Inactivation of *fecE,* encoding the ATP-binding protein component of an Fe"-dicitrate ABC transporter, has previously been shown not to affect Fez' or Fe- transport, or growth on brain heart infu-sion-FCS medium (49). Replacing the *oacA m2* mid region with an ml type did not render the strain toxigenic: neither Tx30a/ KAN nor Tx30a/M1 caused vacuolation in any of the three cell lines tested (Table II and Fig. 3D). VacA levels in water extracts of Tx30a/M1 were not significantly different to those of Tx30a/KAN as determined by ELISA *using* an m2-specific antibody (Ab927) (means 0.32 ± 0.01, n = 4 and 0.35 ± 0,004, n = 2). However, using an ml-specific antibody (Ab929) VacA levels appeared 2-fold higher for Tx30a/M1 (means 0.45 ± 0.01, n = 4 and 0.22 ± 0.01, n = 2, $p < 10^{-8}$). Although it is not possible to directly compare VacA production for these strains by ELISA, owing to antigenic differences in the mid region, it is worth noting that, even if VacA levels were higher in Tx30a/M1 water extracts, no vacuolating activity was observed. This shows that merely replacing the m2 mid region with an ml mid region is insufficient to render a non-toxigenic *vacA* a2/m2 strain toxigenic. Presumably, the type s2 N-terminal extension is sufficient to block toxic activity. Interestingly, strain Tx30a/ Ml, which has a hybrid 32/m1 *vacA* structure, grew similarly to Tx30a and Tx30a/CAT on blood agar and in broth. This con-

firms, in a Tx30a background, our findings in the 60190 and 84-183 backgrounds, that possession of *vacA* with the naturally uncommon s2/m1 structure is not obviously disadvantageous *in vitro.*

vacA from a Toxigenic Strain Is Sufficient to Render a Non-toxigenic Strain Fully Toxigenic—The failure of an ml *vacA* mid region to render tox- strain Tx30a toxigenic could have been because the ml mid region was non-functional in this Tx30a)M1 hybrid. To refute this, we next replaced the s2 signal and promoter regions with sl regions derived from strain 60190, to make a vacA sllml construct in a non-toxigenic Tx30a background. Our second and more important aim in making this hybrid *was* to replace type s2/m2 *vacA* in Tx30a with type slim]. vacA from 60190 to assess to what extent single copy chromosomal complementation with toxigenic vacA conferred activity in a non-toxigenic strain background.

To replace the s2 signal and promoter regions in Tx30a/M1 with sl regions from 60190, we transformed Tx30a/M1 with pCTB2::cat to produce the hybrid strain Tx30a/P1S1M1. Sequence analysis of vacA from this hybrid confirmed that the gene had been replaced with that of the toxigenic strain 60190 with the exception of a 190-bp region from nucleotides 639 to 828 encoding part of the p37 domain of the VacA protein, which was still derived from Tx30a. Water extracts of Tx30a/P1S1M1 induced extensive vacuolation of all three cell types studied, similar to 60190 and controls 60190/CAT and 60190/KAN (Table II and Fig. 3E). VacA levels in the Tx30a/P1S1M1 and 60190/KAN water extracts were also similar as determined by ELISA using Ab929 (means 0.50 ± 0.10 and 0.43 ± 0.01, respectively, $n = 4$ for both, p = This confirms that the ml mid region is functional in a Tx30a background and that *vacA* itself from a toxigenic strain is sufficient to confer full toxigenic activity. Thus differences between tax+ and tox- strains elsewhere on the chromosome do not contribute to differences in VacA toxicity between strains.

The m2 Mid Region from a Non-Toxigenic vacA .82 f na Strain Confers Cell-line Specificity of Vacuolating Activity to a Toxigenic sl I ml Strain—The type m2 mid region of a naturally occurring *vacA* sl/m2 strain has previously been shown to confer specificity of vacuolation such that the strain vacuolates RK13 cells but not HeLa or AGS cells (19). Our previous experiment described above in which we created Tx30a/P1S1 with its sl/m2 structure, suggests that the m2 mid region from an originally non-toxigenic *vacA* s2/m2 strain also confers cell specificity. To prove that the m2 vacA mid region is directly responsible for this effect, we replaced the type ml mid region in strain 60190 with the m2 mid region from Tx30a to create the vacA sl/m2 hybrid strain 60190/1412. Sequence analysis confirmed that the mid region had been replaced with the m2 form downstream of nucleotide 1696. As before we also constructed the control strain 60190/KAN containing the kanamy-cin marker insertion *in fecE.* Water extracts of 60190/M2 and 60190/KAN vacuolated RK13 cells equally (Fig. **4D).** However, in contrast to the control, which also caused extensive vacuo-lation of HeLa and AGS cells, 60190/M2 did not cause vacuo-lation of either of these cell types (Table II). The amount of VacA in water extracts of 60190/M2 appeared nearly 2-fold lower than those of the control 60190/KAN, as determined by ELISA using the m1-specific antibody, Ab929 (means 0.23 -± 0.01 and 0.43 ± 0.01, n - 4 for both, $p < 10^{-4}$), but over 3-fold higher using the m2-specific antibody, Ab927 (means 0.83 0.04 and 0.25 ± 0.005, n = 4 for both, p < 10'). Immunoblot-ting confirmed these findings (data not shown). Given that the ELISA values obtained with each antibody would have been underestimated for VacA alleles of the opposite mid region type, it is likely that the actual VacA amounts for the hybrid and control strains were similar. This experiment shows that the m2 mid region from a non-toxigenic *vacA* s2/m2 strain is functional in determining cell line specificity. Notably also, the vacuolating phenotype of 601901M2 was the same as that obtained for Tx30a/P1S1, showing that the activity of the sllm2 form of *vacA* is independent of strain background.

DISCUSSION

Compared with toxigenic strains of *H. pylori,* non-toxigenic vacA 82./m2 strains are associated with a much lower risk of peptic ulceration and gastric adenocarcinoma (31, 36, 37, 50). In this study we have shown why *vacA* s2/m2 strains are non-toxigenic, the primary determinant being a 12-amino acid N-terminal extension on the VacA protein, which blocks toxin activity regardless of production level or p58 binding region type. Removal of this extension confers toxicity. The m2 *vacA mid region* in non-toxigenic s2./m2 strains is not the cause of non-toxigenicity, but is functional in conferring cell specificity. Chromosomal complementation with single copy *vacA* from a toxigenic *vacA slim* 1 strain shows for the first time that elements outside *eacA* are not needed for full vac-uolating activity.

Mature VacA from toxigenic *vacA* type sl strains has a hydrophobic N-terminal region, which can insert into lipid bilayers (51). In non-toxigenic *vacA* type s2 strains this region is preceded by a 12-amino acid hydrophilic N-terminal extension, which we have shown blocks vacuolating activity. Partially deleting the hydrophobic N terminus of type sl VacA (for example, amino acids 6-27) also blocks vacuolating activity, and, although VacA still forms pores in artificial membranes, they form more slowly and are less anion selective (42). We have previously shown that adding the s2-specific hydrophilic extension to the N terminus of type sl VacA abolishes vacualating activity (41). However, although this slows pore formation in artificial membranes, it does not abolish it nor change the anion selectivity of these pores (43). Thus, it remains unclear whether the hydrophilic N-terminal extension of s2 VacA blocks membrane insertion but that this is not necessary for pore formation in artificial lipid membranes, or whether it acts through another mechanism, far example, through changing the conformation of the active p37 subunit of VacA.

Our key finding is that type s2/m2 VacA is functionally vacuolating once the hydrophilic N-terminal extension is removed. Why *H. pylori* should possess a functional "blocked" form of VacA is a fascinating enigma. One possibility is that the toxin becomes activated *in vivo* through cleavage of this extension. However, if this occurs, it is insufficient to render such strains pathogenic. A second possibility is that VacA possesses an important biological function other than inducing vacuola-tion, which is not blocked by the s2 extension. For example, it may perform other functions ascribed to VacA such as increasing epithelial permeability, stimulating epithelial cell apopto-sis (52), inhibiting antigen presentation (53), or binding to cytoskeletal proteins (54).

Wild-type *H. pylori* strains of *vacA* type s2/m1 have only rarely been isolated (32, 55, 56), and we have identified only one that expresses VacA. Population genetic analyses show that *vacA* structure is characterized by frequent recombination events between vacA from different strains (57-60), so vacA s21m1 structures would be expected to arise *in vivo* as frequently *as 61.1m2* structures. In this study, we constructed the *cowl* s2/m1 structure in both originally toxigenic and originally non-tmdgenic strain backgrounds, and these strains *grew* indistinguishably under laboratory conditions from other strains. Thus, although we speculate that 82/m1 *vacA* offers a selective disadvantage or fails to offer a selective advantage *in vivo,* the nature of this remains unclear.

An important finding in our study is that single copy chromosomal replacement of s2/m2 *vacA in* a non-toxigenic strain with sl/ml var-A from a toxigenic strain confers full vacuolating activity similar to that of the parent toxigenic strain. This shows that production and vacuolating activity of VacA are not dependent on chromosomal elements outside vacA. Furthermore, as full vacuolating activity was conferred without amino acids 639-828 (which are fairly well conserved between vacA alleles) being replaced, we can infer that these residues do not contribute to reduced VacA activity.

Pathogenic strains with the sl/m1 type of VacA have been extensively characterized, including the determination of the complete genome sequence for two such strains (61-63). However, such strains comprise only about 40% of strains isolated from patients undergoing endoscopy (31-38), and this proportion would be expected to be smaller in an unselected population. We have now concentrated on the s2/m2 type of VacA found in non-pathogenic, non-toxigenic strains and the common hybrid sl/m2 type and uncommon hybrid *92.1m1* type (32, 55, 56). We have shown that strains with the type s2 signal region are non-vacuolating due directly to the N-terminal extension on the mature toxin of these strains, The most commonly used test for these strains is an allelic type-specific PCR assay based on detection of this region (31, 46), and our study confirms that this test has a rational biological basis. We have also shown that the mid region of both s2/m2 strains and al/m2 hybrid strains is responsible for cell-specific vacuolation, and this provides validity to the widespread use of mid region allelic type-specific PCR. However, the full significance of *vacA* polymorphism is unlikely to be defined until studies are undertaken in large patient series, and this will not be possible until serum tests are developed which can accurately differentiate between infection with strains expressing the various VacA types.

REFERENCES

1. Paraonnet, J., Friedman, G. D., Vandersteen, D. P.. Chang, Y., Vogelman, J. H., Orentreich, N., and Sibley, R. K. (19911 *New Engl. J. Med.* 325, 1127-1131
2. Parsonnet, *J.*. Hansen, S., Rodriguez, L., Gelb, A., Warnke, R., and Jellum, (19941 *New Engl. J. Med.* 330,1267-1271
3. Warren, J. R., and Marshall, B. (1983) *Lancet* 1,1273-1275
4. Figura, N., Guglielmetti, P., Rossolini, A., Barheri, A-, Cusi, *G.*, Musmanno, R. A., Rossi, M., and Qua ranta, S. 119891,1. *Clin. Microbial.* **27,** 225-226
5. Tee, W., Lambert, *J.* R., and 1-/˜wyer, B. (1995)J, *Clin.* Microbial 35, 1203-1205
6. Cover, T.L., Dooley, C. P., and Ulster, M. J. (1990)Infect. *Inman.* 58,603-610
7. Crabtree, J. E., Taylor, J. D., Wyatt, J. I., Heatley, R. V., Shallcross, T. M., Tompkins, D. S., and Rathbone, B. J. 119911 *Lancet* 338, 332-23.5
8. Weal, *J. F.* L., **van** der Hulot, R. W. M.. Gerrita, Y., Roorda, P., Feller, IA.. Dankert, J., Tytgat, G. N. *J.*, and van der Ende. A. (19981,/.. *Infect. Dis.* ***173,*** 1171-1175
9. Gerhard, M., Lehn, N., Noumayer, N., Bonin, T., Rad, R., Schepp, W., Mi eh Ike, S., Claamen, M., and Prinz, C. (1999) *Prot. Ncitt Arad. Sci. U. S.A.* **96,** 12778-12793
10. Leunk, R. D., Johnson, P. T., David, B. C., Kraft, W. G., and Morgan, D. R. (19861 *J. Meet Microbial*, 28,93-99
11. Marchetti, M., AricO, B.. Burroni, D., Figura, N., Rappuoli, R., and Ghiara, P. (1995) *Science* **267. 1665-1658**
12. Ogura, K., Maeda, S., Nakao. M., Watanabe, T., Tada, M., Kyutoku, T., Yoshida, H., Shiratori, Y., and Omata, M. (20001 *J. Exp. Med.* **192,** 1601-1609
13. Cover, T. L., Tummuru, M. K R., Cao, P., Thompson, S. A., and Blazer, M. J. (19941d. *Biol. Chem.* **269,** 10566-10573
14. Schmitt, W., and Haas, R. (1994) ***Mal*** *Microbial_* 12,307-319
15. Telford, J. L., Ghiara, P., Del l'Orco, M., Comanducci, M., Burroni, D., Bugnoli, M., *Teoce*, M. F., Censini, S., Covacei, A_ Xiang, Z., Papini, E., Montecucco, C., Parente, L., and Rappuoli, R. (19941J. *Exp. Med.* **179,** 1653-1658
16. Phadn is, S. H., liver, D., Janzon, L, Normark, S., and Westblom, T. 1J.119941 *Infect. frames.* **62,** 1557-1568
17. Lupetti, P., Heuser, J. E., Manetti, li., Massari, P., Lanzavecchia, S., 8-ellen, P. L., Dallai. R., Rappuoli. R.. and Telford, J. L. (199611 *Cell Biol.* **183,** 801-807
18. Garner, J. A.. and Cover, T. L. (1996) *Infect. Imman.* **64,** 4197-4203
19. Pagliaccia, C., de Bernard, M., Lupetti, P., Ji, X.., Burroni, D., Cover, T. L., Papini, E., Rappuoli, R., Telford, J. L., and Reyrat, J. (19981 *Proc. Natl. Acad. Sri. U. S. A.* **96,** 10212-10217
20. Wang, H., and Wang, W. (20001 *Biochem. Btophys. Res. Common.* **278, 449-454**
21. Yc, D., Willhite, D. C-, and Blanke, S. 8.119991 *J. Biol. Chem.* 274,9277-9282
22. Szabo. I., Brutsche. 8., Tombola, F., Mosehioni, M., Satin, B., Telford, J. L., Rappuoli, R., Monteeucco, C., Papini, E., and Zoratti, M. (1999) *EMBO* **18.** 5517-5527
23. Tombola, F., Oregna, F., Brutsche, S., Saab[, I., Del Giudice, G., Rappuoli, R., Montecucco, C., Papini, E., and Zoratti, M. (1999) *FEBS Lett.* **460,** 221-225
24. Cover, T. L., Reddy, L. Y., and Blamer, M. J. 11993) *Infra. Imatan.* **61,** 1427-1431
25. Molinari, M.. Galli, C., Norais, N., Tel ford, J. L.. Rappuoli, R., Luzio, J. P., and Montecucco, C. (19971 *J. Riot. Chem.* **272,** 25339-25344
26. Papini, E., Bugnoli, M.. de Bernard, H. Figura, N., Rappuoli, R., and Montecucco, C. (1993) *Mn!. Microbial. 7*, 323-327
27. Papini, E., de Bernard. M.. Bugnoli, M., Milia, E., Rappuoli, IL, and Monteeetvo, C. (19931 *FEMS Microbial. Lett.* **113,** 155-160
28. Papini, E., de Bernard. M., Milia, E., Bugnoh, M., Zerial, M., Rappuoli, R., and Montecucco, C. (1994) Proc. *Natl. Acad. Sci. U. S. A.* 91,9720-9724
29. Papini, E., Gottardi, E., Satin, B., de Bernard, M., Maasari, P., Telford, J., Rappuoli, R., Sato, S. B., and Montecucco, C. 119961,1. *Med_ Microbial.* **13,** 84 -89
30. Ricci., V., Sommi, P., Fiona, R., Romano, M., Soleia, E., and Ventura, U. (19971 *J. Pathol.* **183,** 453-459
31. Atherton, J. C., Can, P., Peek, R. M., Tummuru, M. K. R., Blaser, M. J., and Cover, T. L 11995)1. *Biol. Chem.* **270,** 17771-17777
32. Letley, D. P., Lastoviea, A., Louw, J. A., Hawkey, C. *J.*. and Atherton., J. C. (19991J. *Ulm Microbial.* 57,1203-1205
33. Atherton, J. C., Peek, R. M., Jr., Tham, K. 1`., Cover, T. L., and Rinser, M. J. (19971 *Gastroenterology* 112,92-99
34. van Doom, L J., Figueiredo, C., **Raisin,** R., Jan.nee, G., van Asbroeck, M., Sousa, J. C., Carneiro, F., and Quint. W. G. V.119981,1. *Clin. Microbial.* 36, 1271-1276
35. Strobel, S., Bereswill, S.. Balig, P., Allgaier, P., Sonntag, H., and Kist, M. (19951J. Clio. *Microbial.* **86,** 1286-1289
36. Rudi, J., Kolb, *C.*, Maiwald, M., Kuck, D., Sieg, A., Calle, P. R., and Stremmel, W. (1998) *J. Clin. Microbial. $6*, 944-948
37, Basso, D., Navaglia, F., Brigato, L. Pies, M. G., Tama, A., Green, E.. Di Mario, F., Galeotti, F., Roveroni, G., Corsini, A., and Flebani, Vii. (19981 *Gut* 43, 182-186
38. Han, 8., Schreiber, 11., Shalcdi, S., **thou,** M., and Maeurer, M. J. (1998) *Can. Diagn. Lab. Immunol.* 8, 139-145
39. Forsyth, M. **El.,** Atherton, J. C., Rinser, M. ***J.,*** and Cover, T. L.119981 *Infect. finnan.* 66, 3088-3094
40. de Bernard. M., Burroni, D., Papini, E., Rappuoli, R., Telford, J., and Montecucco, C. (19981 *Infect. Immun.* **66, 6014-6016**
41. Letley, D. P., and Atherton, J. C. (200011. *Bartered.* **162,** 3278-3280
42. Vinion-Dubiel, A. D., McClain, M. S., Czajkowsky, D. M., Iwamoto, H., Ye, D., Cao, P., Schraw, W., Szabo, G., Blanks, S. R., Shao, Z., and **Coney,** T. L. (1999)1 *Biol. Chain.* 274, 37736-87742
43, McClain, M. S., Cao, P., Iwamoto, H., Vinion-Dubiel, A. D., Szabo, G.. Shan,. and Cover, T. L. 1200111 *Bacteria.* **183,** 6499-6508
44. Ji, X., Fernandez, T., Burroni, D., Pagliaccia, C., Atherton, J. C., Reyrat, J., Rappuoli, R., and Telford, *J.* L. 12000) *Infect, Immun.* **60,** 3754-3757
45. Atherton. J. C. (19971 in *Methods in Molecular Medic-Ma, Hellennader pylori Praloe-als* (Clayton, C. L., and Mobley, H. L. T., ids) pp. 133-145, Humana Press, Totowa, NJ
46. Atherton, *J.* C., Cover, T. L., Twella, R. J., Morales, M. R., Hawkey, C. J.. and Blaser, M. J.1199911. *Clin. Microbial*, **37,** 2979-2982
47. Cover, T. L., and Blamer, M. J. (1992) *J. Bid. Chem.* **287,** 10570-10575
48. Wang, H.-J., Chang, P. C. L., Eno. C.-H., Tzeng, C.-S., and *Wang, W.-C. (19981 Riochein. Riophys. Res.* Commun. **230,** 397-402
49. Velayudhan, J., Hughes, N. J., MeCoint, A. A., Bagahaw, J., Clayton, C. L., Andrews, S. C., and Kelly, D. J. (20001Mol. *Microbial. 37*, 274-286
50. Michlke, S., Kirsch, C., Agha-Amiri, K., Gunther, T., Lehn, N., Malfertheiner, P., Stolte, M., Ehninger, G., and Bayerdtlrffer, E. (2000) *Int. J. Cancer* **87,** 322-327
51. McClain, M. S., Can, P., and Cover, T. L. (2001) *Infect. !amain.* 69, 1181-1184
52. Kuck, D., Kolmerer, B., !king-Kermit. C., Krammer, P. H., Stremmel, W., and Budi, J. (2001) *Infect. Inman*, **69,** 5080-5087
53. Molinari, M., Sabo, M., Galli, C., Norais, N., Rappuoli, R., Lanzavecchia, A., and Manteruceo, C. (1998)J. *Exp. Med.* 187,13.5-140
54. de Bernard, M., Moschioni, M., Napolitani, G., Rappuoli, R., and Menlecueco, C. 12000) *EMBO J. 19*, 48-56
55. Morales-Espinosa, R., Castillo-Rojas, G., Gonzalez-Valencia, G., de Leon, S. P., Cravioto, A., Atherton, J. C., and Lopez-Vidal, Y.1199911. *air'. Microbial.* 37,3001-3004
56. Martinez, T. A., Gonzalez, C. C., Kawaguchi, P. F., Montoya, R., Corvalart, A., Madariaga, *J.*. Roa, *J., Garcia.* C. A, Salgado, F., Solar. H., and Palma. M. (2001) Rev. *Mad. Chile* **129,** 1147-1153
57. Atherton, J. C., Sharp, P. M., Cover, T. L., Gonzalez-Valencia, G., Peek, R. M., Jr., Thompson, S. A., Hawkey, C. J., and Blazer, M. J. (1998) *Curr. Microbial.* **39,** 211-218
58. Achtman, M., Azusa, T., Berg, D. E., Ito, Y., Morelli, G., Pan, Z.-J., Suerbaum, S., Thompson, a A., van der Ende, A., and van Doom, L. J. *(1999) Mod. Microbial.* **32,** 459 -470
59. Suerbaum, S., Maynard Smith, J., Bapumia, K., Morelli, G., Smith, N. H., Kunstmann, E., Dyrek. I., and Achteuut, M. (19981 Proc. *Nag Acad.. Sri. U. S.* **A. 95,** 12619-12624
60. Ji, X., Frati, F., Barone, S., Paggliacia, C., Burroni, D., Xu, Rappuoli, R., Reyrat, J. M., and Telford, J. L. 20021FEMS *Microbial- Lett.* **206, 253-258**
61. Atherton, J. C., Cover, T. L., Papini, E., and Telford, J. L. (20011 in *Helico-barter pylori: Physiology and Genetics* (Hazel), S. L., edl pp. 97-110. ABM Press, Washington, D. C.
82. Alm, R. A., Ling, L. S. L., Moir, D. T., Ring, B. L., Brown, E. D., Doig, P. C., Smith, D. R., Noonan, B., Guild, B. C., deJonge, B. L, Carmel, G., Tummino, P. J., Caruso, A., Uria-Nickelson, M., Mills, D. M.. Ives, C.,

Gibson, R., Merberg, D., Mills, S. D., Jiang, Q., Taylor, D. E., Vovis, G. F., and Trost, T. J. (1999) *Nature* **397,** 176–180
63. Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L. X., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., McKenney, K., Fitzegerald, L. M., Lee, N., Adams, M. D., Hickey, E. K., Berg, D. E., Gocayne, J. D., Utterback, T. R., Peterson, J. D., Kelley, J. M., Cotton, M. D., Weldman, J. M., Fujii, C., Bowman, C., Watthey, L., Wallin, E., Hayes, W. S., Weidman, J. M., Borodovsky, M., Karp, P. D., Smith, H. O., Fraser, C. M., and Venter, J. C. (1997) *Nature* **388,** 539–547
64. Pérez-Pérez, G. I., and Blaser, M. J. (1987) *Infect. Immun.* **55,** 1256–1263

CHAPTER 5

PAIRED CYSTEINE RESIDUES ARE REQUIRED FOR HIGH LEVELS OF THE *Helicobacter pylori* AUTOTRANSPORTER VacA

Letley, D. P., J. L. Rhead, K. Bishop and J. C. Atherton (2006)

Microbiology **152**:1319-1325

CHAPTER 6

A NEW *Helicobacter pylori* VACUOLATING CYTOTOXIN DETERMINANT, THE INTERMEDIATE REGION, IS ASSOCIATED WITH GASTRIC CANCER

Rhead*, J. L., D. P. Letley*, M. Mohammadi, N. Hussein, M. A. Mohagheghi, M. E. Hosseini and J. C. Atherton (2007)

Gastroenterology **133**:926–936

*These authors contributed equally

CHAPTER 7

GENERAL DISCUSSION

7.1 Natural *vacA* polymorphism

The mosaic structure of *vacA* suggests recombination occurs between *vacA* alleles, most likely during multiple infection, or transient infection with another strain. Such recombination suggests the existence of all combinations of *vacA* signal and mid-region type. For a long time, the *vacA* s2/m1 allele remained elusive despite many typing studies based on clinical isolates from *H. pylori*-infected populations around the world. It seemed unlikely that this allelic variant would not arise through recombination, and so it was suggested that *vacA* s2/m1-type strains were either non-viable, or the allele conferred a selective disadvantage (Atherton *et al.*, 1995). Our discovery of a *vacA* s2/m1-type strain among a collection of clinical isolates from South Africa showed for the first time that such alleles can arise naturally, and strains with this *vacA* genotype can colonise the human gastric mucosa (Letley *et al.*, 1999 [Chapter 2]). Since this first report, *vacA* s2/m1-type strains have been isolated from several other populations including Mexico (Garza-Gonzalez *et al.*, 2004; Morales-Espinosa *et al.*, 1999), Chile (Martinez *et al.*, 2001), Argentina (Catalano *et al.*, 2001), Costa Rica (Con *et al.*, 2007), Cuba (Torres *et al.*, 2009), Saudi Arabia (Ismail *et al.*, 2001), Ethiopia (Asrat *et al.*, 2004), Nottingham UK (Bebb *et al.*, 2003), Italy (De Francesco *et al.*, 2009) and further isolates from South Africa (Kidd *et al.*, 1999). The discovery of *vacA* s2/m1 strains from diverse geographical regions supports the hypothesis that *vacA* mosaicism arises from recombination between *vacA* alleles. However, despite these reports, the *vacA* s2/m1 allele remains a rarity in all populations studied to date, and the reason for this is unclear. We and others have generated artificially constructed *vacA* s2/m1 alleles, and strains expressing this form of the toxin do not show any reduced viability *in vitro* (Letley and Atherton, 2000 [Chapter 3]; Letley *et al.*, 2003 [Chapter 4]; McClain *et al.*, 2001b). However, this does not rule out a selective disadvantage conferred by the s2/m1 form of the toxin *in vivo*, particularly if newly arising s2/m1 recombinants are competing with their parental strains in the context of a multiple infection. Functionally, the s2/m1 form of the toxin is nonvacuolating (Letley and Atherton, 2000 [Chapter 3]; Letley *et al.*, 2003 [Chapter 4]), and forms anion channels in lipid bilayers more slowly than the s1/m1 form (McClain *et al.*, 2001b). The significance of VacA channels *in vivo* is not clear, but they may act as urea permeases, releasing urea from host cells to aid acid resistance (Tombola *et al.*, 2001a), or generally increase the availability of

nutrients for *H. pylori* growth. Furthermore, while s1 forms of VacA may also promote nutrient release by weakening tight epithelial cell junctions (Papini *et al.*, 1998; Pelicic *et al.*, 1999), it has not been determined whether s2 forms of the toxin also possess this activity. Therefore, it is feasible that the altered channel properties of s2 VacA could affect *H. pylori* colonisation efficiency. However, it seems unlikely that strains expressing s2 forms of the toxin have a significant growth disadvantage *in vivo* given that *vacA* s2/m2 strains are common in many European and USA populations. Indeed, one study has shown that s2-type strains colonise with higher density than s1 strains (van Doorn *et al.*, 2000), although this could be due to more virulent s1 strains causing greater inflammation, rather than any enhanced growth ability of s2 strains. Furthermore, it is reasonable to assume that s2/m1 recombinant strains arising during multiple infection may benefit *in trans* from the VacA channels secreted by closely neighbouring *vacA* s1-type strains also present. While there has been extensive focus on the clinical aspects of *vacA* polymorphism, with particular emphasis on the pathogenic role of the s1/m1 form of the toxin, the role of the nonpathogenic s2 forms of VacA on the growth and survival of *H. pylori* in its gastric niche still remains to be determined. Once a clear understanding of how VacA benefits *H. pylori* colonisation has been established, the reason for the rarity of the s2/m1 allele may become more apparent.

Our finding that the *vacA* s1b-allele was predominant among a small collection of *H. pylori* strains from South Africa (Letley *et al.*, 1999 [Chapter 2]) was one of the first reports of *vacA* allelic diversity among *H. pylori* strains isolated from the African continent. The predominance of the s1b-allele in South Africa was later confirmed for a larger number of strains (Kidd *et al.*, 1999). In both studies, s1b strains were more commonly m1-type than m2. *vacA* s1b/m1 was also found to be the predominant allelic variant among strains isolated from Zambia, also located within southern Africa (Raeiszadeh *et al.*, 2003). While *vacA* s1a-type strains have been shown to be the predominant strain among *H. pylori*-infected patients from Sudan, located in the northeastern region of Africa, multiple infection with an s1b-type strain was very common (El Mahadi *et al.*, 1999). In neighbouring Ethiopia, s1/m1 strains have been shown to be the most common (Asrat *et al.*, 2004), while the s1/m2 allele was predominant among Nigerian strains from west Africa (Smith *et al.*, 2002), although neither of these studies determined the subtype of the s1-strains identified. In contrast to the predominance of s1

strains in these studies, s2/m2 strains were found to predominate among *H. pylori* isolates from the north African countries Egypt (Van Doorn *et al.*, 1999) and Tunisia (Ben Mansour *et al.*, 2010). However, among the s1 strains in the Tunisian study, the s1b allele was the most common, as observed for other African countries (Ben Mansour *et al.*, 2010). Interestingly, a study of *vacA* diversity among ethnic groups in Houston, Texas found that the *vacA* s1b/m1 allele was also predominant among strains from the black population, in contrast to the white population which were more commonly s1a/m2 (Yamaoka *et al.*, 2000b).

Although *H. pylori* infection rates are very high in Africa, the incidences of peptic ulcer disease and gastric cancer are surprisingly low, an observation which has been referred to as the 'African Enigma' (Holcombe, 1992). As *vacA* s1a alleles had earlier been shown to be more strongly associated with duodenal ulcer disease and greater inflammation than s1b alleles among strains from the United States (Atherton *et al.*, 1997), we speculated that the rarity of s1a-type strains in South Africa may contribute to the low incidence of *H. pylori*-related disease (Letley *et al.*, 1999 [Chapter 2]). Later studies have also shown *vacA* s1a-type strains to be more strongly associated with peptic ulcer disease (Catalano *et al.*, 2001; Diaz *et al.*, 2005) and gastric cancer (Qiao *et al.*, 2003) than the s1b allelic form, further suggesting that *vacA* s1b-type strains may be less pathogenic. We have shown for one s1b-type strain that replacing the promoter and signal region with the s1a form increases VacA production (Letley *et al.*, 2003 [Chapter 4]), and Atherton *et al.* (1997) reported that vacuolating activity was significantly higher for s1a/m1 strains than s1b/m1 strains, although whether this was due to reduced VacA production among the s1b strains was not determined. The s1b allele has also been shown to be the predominant *vacA* type among *H. pylori* isolates from the Iberian Peninsula (Portugal and Spain), and Southern and Central America (Van Doorn *et al.*, 1999), where gastric cancer incidence is markedly higher than in Africa (Ferlay *et al.*, 2010). This emphasises that multiple factors most likely contribute to the low incidence of gastric disease in Africa. For example, global variation in gastric cancer incidence has been associated with allelic differences in *cagA*, and the frequency of *cag*-negative strains within the population (Yamaoka *et al.*, 2008). A study of *H. pylori* strains from high and low gastric cancer risk regions of Colombia showed that the geographical origin of the strain was a determinant: strains of African origin were only found in the low risk region, and were

associated with reduced severity of premalignant histological changes compared to strains of European origin (de Sablet *et al.*, 2011), highlighting that genetic differences may make African *H. pylori* strains less virulent. Concurrent enteric infection with helminths may also contribute to geographical variations in *H. pylori*-related disease incidence by modulating the host immune response (Fox *et al.*, 2000). Additionally, environmental factors such as diet, as well as host genetic differences would be expected to play a role. Finally, the existence of an 'African Enigma' has itself been questioned, as a more recent review of published endoscopic studies in African populations found no evidence for discordance between *H. pylori* prevalence and gastric disease (Agha and Graham, 2005).

7.2 Relationship between *vacA* polymorphism and vacuolating activity

When *vacA* from a nontoxigenic strain was first characterised it became apparent that allelic variation within the gene encoding the cytotoxin was associated with differences in vacuolating activity observed between strains *in vitro*: nontoxigenic strains invariably possessed an s2/m2 form of *vacA*, while s1/m1-type strains often showed more vacuolating activity than s1/m2-type strains (Atherton *et al.*, 1995). However, how this natural polymorphic variation in *vacA* led to variation in strain toxigenicity required further characterisation. For example, structural differences in the cytotoxin encoded by this allelic variation could directly affect its activity, its ability to bind to epithelial cells or its interaction with putative host cell factors required for vacuolation to occur. Additionally, variation within the signal region could potentially act as a marker for differences within the promoter region, or affect signal sequence processing efficiency during secretion of the toxin across the cytoplasmic membrane, both of which might affect the production of the mature toxin. It was also unclear whether other bacterial factors contributed to the vacuolating phenotype of *H. pylori*, which may be variable between strains. The published papers presented in this thesis describe the effects of signal and mid-region polymorphisms on the vacuolating activity of the toxin. They also describe a new polymorphic determinant of vacuolating activity, the intermediate region. A summary of the vacuolating activity observed for the isogenic *vacA* mutants and their wild-type control strains is shown in Table 1.

Table 1 *vacA* allelic mutant and control strains studied in this thesis, and theirvacuolating activity

Mutant strain	*vacA* allelic type	Vacuolating activity[A]			Ref
		HeLa	AGS	RK13	
60190/CAT or 60190/KAN	s1 / i1 / m1[B]	+	+	+	Letley *et al*., 2003
60190 *vacA*::N(s2)	s2 / i1 / m1	-	-	-	Letley & Atherton 2000
60190/P2S1	s1 / i1 / m1	+	+	+	Letley *et al*., 2003
60190/P2S2	s2 / i1 / m1	-	-	-	Letley *et al*., 2003
60190/M2	s1 / i1 / m2	-	-	+	Letley *et al*., 2003
60190/A2	s1 / i2(cluster A) / m1	+	+	+	Rhead *et al*., 2007
60190/B2	s1 / i2(cluster B) / m1	-	-	+	Rhead *et al*., 2007
60190/C2	s1 / i2(cluster C) / m1	-	-	+	Rhead *et al*., 2007
Tx30a/CAT or Tx30a/KAN	s2 / i2 / m2[B]	-	-	-	Letley *et al*., 2003
Tx30a/P1S1	s1 / i2 / m2	-	-	+	Letley *et al*., 2003
Tx30a/N1	s1 / i2 / m2	-	-	+	Letley *et al*., 2003
Tx30a/M1	s2 / i1 / m1	-	-	-	Letley *et al*., 2003
Tx30a/P1S1M1-A	s1 / i1 / m1	+	+	+	Rhead *et al*., 2007
Tx30a/P1S1M1-B	s1 / i2 / m1	-	-	+	Rhead *et al*., 2007
93-67/CAT	s1 / i1 / m2[B]	ND	ND	+	Rhead *et al*., 2007
93-67/i2-i1	s1 / (i2-i1) / m2	ND	ND	+/-	Rhead *et al*., 2007
93-67/i1-i2	s1 / (i1-i2) / m2	ND	ND	+/-	Rhead *et al*., 2007
J226/KAN	s1 / i2 / m2[B]	ND	ND	-	Rhead *et al*., 2007
J226/i1-i2	s1 / (i1-i2) / m2	ND	ND	+/-	Rhead *et al*., 2007

[A] The presence (+) or absence (-) of cytoplasmic vacuolation as determined by light microscopy is shown. +/- indicates a quantitative reduction in vacuolation relative to 93-67/CAT as determined by counting vacuolated cells in random fields. ND, not determined.

[B] *vacA* type represents the wild type allele for this strain.

7.2.1 Signal region polymorphism

An insertion in the s2-form of VacA had been shown to result in a different signal sequence processing site leading to an altered mature N-terminus containing a 12 amino acid extension relative to the s1 form (Atherton *et al.*, 1995). To examine whether this N-terminal extension affected the vacuolating activity of the mature secreted toxin, we constructed an isogenic mutant of the strongly toxigenic s1/m1 strain 60190, which contained the same 12 amino acid extension as the non-toxigenic s2/m2 strain Tx30a (Letley and Atherton, 2000 [Chapter 3]). During the course of this study, other mutagenesis strategies on the toxigenic s1/m1 form of the toxin were published, which showed that deletions at the N-terminus of the mature protein had a profound effect on vacuolating activity (de Bernard *et al.*, 1998a; Vinion-Dubiel *et al.*, 1999; Ye *et al.*, 1999; Ye and Blanke, 2000). However, it was still not clear whether the natural extension to the N-terminus of s2 forms of the toxin would similarly disrupt toxin activity. Indeed, characterisation of our s2-extension mutant showed that vacuolating activity was blocked by the addition of these 12 amino acids resulting from the different signal processing site in s2-type VacA (Letley and Atherton, 2000 [Chapter 3]). This finding was later confirmed by McClain *et al.* (2001b) who also demonstrated that the s2-extension to the N-terminus reduced the rate of toxin channel formation in artificial lipid bilayers, although did not alter its anion selectivity. The mature N-terminus of the toxigenic s1-form of VacA represents one of the only hydrophobic regions of the protein, and we speculated that the hydrophilic nature of the s2-specific 12 amino acid extension may account for the alteration in vacuolating activity observed (Letley and Atherton, 2000 [Chapter 3]). Indeed, the hydrophobic N-terminus contains *GXXXG* motifs which have been shown to promote transmembrane dimerisation of VacA monomers, and are essential for vacuolating activity (McClain *et al.*, 2001a; McClain *et al.*, 2003). It has been proposed that the *GXXXG* motifs from adjacent VacA monomers associate to form a transmembrane channel (Kim *et al.*, 2004). Therefore it is likely that the addition of hydrophilic residues to the hydrophobic N-terminus would impede transmembrane interactions between monomers, affecting channel formation.

While we had shown that the hydrophilic s2-extension could block the vacuolating activity of the s1/m1 form of VacA, it could not be ruled out that other factors contributed to the lack of vacuolation observed for s2/m2-type strains. To address this, we constructed s-

region hybrid constructs and an s2-extension deletion mutant in the non-toxigenic s2/m2 strain, Tx30a (Letley *et al.*, 2003 [Chapter 4]). We showed that removing the hydrophilic 12 amino acid extension could restore vacuolating activity to an otherwise s2/m2-type strain, albeit in a cell-type-specific manner similar to that observed for natural s1/m2 strains (Letley *et al.*, 2003 [Chapter 4]). This study also demonstrated that full vacuolating activity could be established in a nontoxigenic, s2/m2-type strain background by replacing both the signal and mid regions with the s1 and m1 forms, respectively. This showed for the first time that *vacA* allelic variation was the only factor responsible for the lack of vacuolating activity in nontoxigenic strains. This finding supports the extensive use of *vacA* allelic typing by PCR to characterise *H. pylori* clinical isolates, especially given the association between peptic ulcer disease, and the ability of the infecting strain to cause cytoplasmic vacuolation of epithelial cells *in vitro* (Figura *et al.*, 1989; Tee *et al.*, 1995).

Our analysis of signal region hybrids between the toxigenic s1/m1 strain 60190 and the nontoxigenic s2/m2 strain Tx30a also revealed that differences upstream of the *vacA* open reading frame affected the level of VacA produced: the s1 upstream region increased production of the s2/m2-type toxin, and the s2 upstream region decreased production of the s1/m1 form of VacA (Letley *et al.*, 2003 [Chapter 4]). While our studies did not discriminate between transcriptional and translational differences, Forsyth *et al*. (1998) earlier showed that s1-type strains had higher VacA transcription levels than s2-type strains. While we have shown that reduced VacA production in s2/m2 strains does not account for their lack of toxicity, we have recently found that among s1-type strains, higher *vacA* transcription levels *in vivo* are associated with more severe gastric inflammation and atrophy (C. Masters *et al*., manuscript in preparation).

7.2.2 Mid-region polymorphism

Pagliaccia *et al*. (1998) previously showed that the naturally-occuring *vacA* s1/m2 strain 95-54, which did not induce vacuolation of HeLa cells *in vitro*, was able to vacuolate another cell line, RK13, derived from rabbit kidney epithelial cells. We observed similar cell-specificity for our isogenic *vacA* s1/m2 hybrids constructed in both toxigenic and nontoxigenic parental strain backgrounds. The cell-specific vacuolating phenotype of strain 95-54 was attributed to a

defect in VacA binding to HeLa, but not RK13 cells (Pagliaccia *et al.*, 1998). Interestingly, the majority of the polymorphic differences between m1 and m2-type VacA correspond to residues residing on the surface of the p55 domain's 13-helix structure (Gangwer *et al.*, 2007), which could potentially interact with host cell receptors. Several receptors have been implicated in the binding of m1-type VacA to epithelial cells, including the receptor-like protein tyrosine phosphatases (RPTP) LI] and 13 (see section 1.4.3). The m2-form of VacA could bind to a different receptor to the m1 form, which is either lacking or expressed at a very low level in HeLa cells, or the two forms may bind with different affinity to the same receptor, which is either expressed at different levels in different cell lines, or is polymorphic. To address this, De Guzman *et al.* (2005) studied the interactions between host cell proteins and the m2-form of VacA. Using immunoprecipitation, they showed that, like the m1 form, m2-type VacA bound to both RPTPLI] and RPTP13, and that RPTPLI] alone was sufficient to confer sensitivity to both m1 and m2-forms of the toxin. Furthermore, while HeLa cells express only RPTPLI], the receptor is expressed at a lower level than in RK13 cells, and appears to be post-translationally modified affecting its ability to bind m2-type, but not m1-type VacA (De Guzman *et al.*, 2005). A lower expression of RPTPLI] most likely explains the reduced binding of m1-type VacA to HeLa cells compared to RK13 cells (Pagliaccia *et al.*, 1998). We also found AGS cells to be insensitive to vacuolation by m2-type VacA (Letley *et al.*, 2003 [Chapter 4]) and these similarly lack RPTP13 and have a modified form of RPTPLI] which differs to both HeLa and RK13 cells (De Guzman *et al.*, 2005).

While RPTP13 does not play a role in the vacuolation of the cell lines used in our study, it has been shown to have an essential role in the pathological effects of the toxin in mice (Fujikawa *et al.*, 2003). An extracellular domain of RPTP13 has been shown to be involved in VacA binding, and glycosylation of this region appears to be important (Yahiro *et al.*, 2004). Skibinski *et al.* (2006) have suggested that the cell-specific phenotype attributed to different mid-region types is independent of the RPTP13 receptor, based on the observation that HL-60 cells remained more sensitive to the m1-form of the toxin than an isogenic m2-hybrid form, even when treated with PMA which increased the expression of RPTP13. However, PMA-treatment did greatly increase sensitivity of HL-60 cells, and to some extent HeLa cells, to the m2-form of VacA, consistent with both forms binding to RPTP13. However, it is difficult to draw

conclusions about the role of RPTPI3 from these studies as PMA treatment could have affected the expression of other putative VacA receptors, including RPTPLI], influencing the amount of vacuolation observed. It is possible that both RPTPLI] and RPTPI3 play an active role in the pathological effects of VacA during *H. pylori* infection in humans, and it will be important for future studies to determine the existence of natural variation in post-translation modification of these proteins among populations, the effect these have on the binding of the different forms of VacA, and the consequences for disease outcome.

7.2.3 Intermediate region polymorphism

During our studies on the effect of signal and mid-region polymorphisms on vacuolating activity we identified a new determinant of vacuolating activity located within the p33 domain which we called the intermediate region (i-region) (Rhead *et al.*, 2007 [Chapter 6]). This finding has most significance among *vacA* s1/m2-type strains where heterogeneity in i-region type exists, and we showed that among such strains, only i1 variants vacuolated RK13 cells (which are sensitive to m2-type VacA). We confirmed this using an isogenic mutant approach. There are several ways in which i-region polymorphisms could influence the vacuolating phenotype of the toxin, including affecting its ability to bind to epithelial cells; to form oligomeric structures; to become internalised; or perturb the formation or activity of the anionic channel, and some of these mechanisms have been discussed previously (Rhead *et al.*, 2007 [Chapter 6]). While our finding that isogenic s1/i2/m1 variants (a type rarely found among clinical isolates) had a cell-specific vacuolating phenotype similar to s1/m2-type strains suggests a role in VacA binding, other mechanisms cannot be ruled out. Pagliaccia *et al*. (1998) observed that RK13 cells bound more of the m1-type toxin than HeLa cells, and De Guzman *et al*. (2005) showed that RK13 cells express higher amounts of the RPTPLI] receptor, and vacuolate more strongly than HeLa cells. Thus RK13 cells are more sensitive to vacuolation by s1/m1 VacA than HeLa cells, and consequently the reduced toxin activity of s1/i2/m1 VacA through whatever mechanism, may be more apparent for HeLa cells than RK13. More recently we have found quantitative differences in the ability of wild type s1/i1/m1 VacA and s1/i2/m1 isogenic variants to vacuolate RK13 cells, indicating the effects may not be cell-specific (unpublished data).

At present, the only VacA crystal structure data available is for the p55 domain. However, secondary structure predictions, and the shape of VacA monomers in electron microscopy maps of the oligomeric structure, suggest that a large proportion of the p33 domain may extend the 13-helix fold found in the p55 domain (Gangwer *et al.*, 2007). The i-region lies within this predicted 13-helix fold, and it is conceivable that polymorphic differences occur on the surface of this structure, as is the case for mid region polymorphisms (Gangwer *et al.*, 2007), where they could influence interactions with either host cell receptors, or with neighbouring VacA monomers affecting oligomerisation.

Using a random mutagenesis approach, McClain *et al*. (2006) identified several amino acid substitutions which abolished the toxin's ability to vacuolate HeLa cells. While most of these mutations were located within the well characterised hydrophobic N-terminus of the p33 domain, four affected residues within the C-terminal half of p33, predicted to form a 13-helix fold. Interestingly, one mutation, T210A, lay within the i-region between cluster B and C. However, the importance of this residue was difficult to ascertain, as while it abolished vacuolating activity when present in recombinant VacA expressed in *E. coli*, it had no effect when introduced into the chromosomal *vacA* gene in *H. pylori*. The authors suggest this anomaly may be due to differences in the way the toxin folds when expressed in its mature form in *E.coli* compared with its native, precursor form in *H. pylori*. Another mutation, S246L, was located just 23 residues down from the i-region. This substitution abolished vacuolating activity in both the *E. coli* recombinant system and naturally in *H. pylori*. The mutated toxin still associated into oligomeric structures, and formed channels with the same anion selectivity as wild type VacA. However, subtle differences in the channel properties of the mutated toxin existed, as evidenced by a reduced ability to depolarise intact cells, and an altered rate of channel formation on lipid bilayers at positive, but not negative, membrane potentials. Thus, it is feasible that i-region polymorphisms could affect the channel properties of the toxin in a similar manner.

7.3 Association between *vacA* intermediate region type and *H. pylori*-related gastric disease

Having identified the i-region as a new determinant of VacA toxicity and shown by sequence analysis that both i1 and i2-types were common among a selection of Western *H. pylori* strains, we devised a simple PCR-based typing system that could be used to study other populations to assess the association between i-region type and disease (Rhead *et al.*, 2007 [Chapter 6]). Using this typing system, we found that among a group of *H. pylori*-infected patients from Iran, i1-type strains were significantly associated with gastric cancer. Further more, multivariate analysis suggested that i-region type may be a better marker of VacA-associated disease than the other polymorphic regions studied to date. Our PCR typing system has now been successfully used to determine the i-region type of *H. pylori* clinical isolates from Italy (Basso *et al.*, 2008), Greece (Panayotopoulou *et al.*, 2010), Iran (Hussein *et al.*, 2008), Taiwan (Sheu *et al.*, 2009), Pakistan (Yakoob *et al.*, 2009), South Korea (Jang *et al.*, 2010), and strains from the US, Columbia, South Korea and Japan (Ogiwara *et al.*, 2009). Collectively, these studies confirm our findings that virtually all s1/m1 strains are i1-type and s2/m2 strains are i2-type, with s1/m2-type strains being variable in their i-region status, presumably based on whether recombination occured before or after the i-region when that particular *vacA* allele arose.

The i-region typing system we described discriminates *vacA* alleles based on polymorphic differences within Cluster C. Recently, Chung *et al*. (2010) determined the nucleotide sequence of the entire *vacA* i-region of 120 clinical isolates from Chinese, Turkish, Uruguayan and Afro-American patients. They constructed a phylogenetic tree based on the complete i-region sequence which confirmed the assessment of i-region type based solely on sequence polymorphisms of both clusters B and C. As we found, while the majority of strains were the same i-region type for both cluster B and C, a few strains showed discordance in i-type between the two clusters, and these were refered to as i3-type strains. The majority of these i3-type sequences were i1-type for cluster B and i2-type for cluster C, the same recombinant type we observed for two of the strains in our study (Rhead *et al.*, 2007 [Chapter 6]). These i3-type strains originated from the same main phylogenetic branch as the i2-type strains, which would agree with their classification as i2-type based on our PCR-typing of the

cluster C polymorphisms. Interestingly, the i1-type strains formed country-specific clusters within the tree, while i2-types were more homogeneous. This parallels the observation that mid region sequences are more conserved among m2-type strains than m1-type strains (Atherton *et al.*, 1999b; van Doorn *et al.*, 1998b).

Our finding that i1-type strains were associated with increased gastric cancer risk among an Iranian population, has similarly been shown for *H. pylori*-infected patients from Italy (Basso *et al.*, 2008), Pakistan (Yakoob *et al.*, 2009) and the Americas (US and Colombia)(Ogiwara *et al.*, 2009). Furthermore, associations between i1-type strains and duodenal ulcer (Basso *et al.*, 2008), gastric ulcer (Hussein *et al.*, 2008) or both (Ogiwara *et al.*, 2009; Yakoob *et al.*, 2009) have been reported, as has an association with severe chronic inflammation and marked active gastritis (Panayotopoulou *et al.*, 2010). In Taiwan (Sheu *et al.*, 2009), South Korea (Jang *et al.*, 2010; Ogiwara *et al.*, 2009) and Japan (Ogiwara *et al.*, 2009), virtually all the strains studied were i1-type, meaning no association with disease could be shown, although it is interesting to note that the incidence of gastric cancer is high in these regions. In many of these studies, associations between gastric disease and other *vacA* allelic types (s1 and m1) and *cagA* status (positivity or number of phosphorylation motifs) were described, as were associations between these other known markers of *H. pylori* pathogenicity and the presence of the i1 allele. To address the relative importance of the i-region as a marker of gastric disease compared to other *vacA* allelic types and the presence of *cagA*, we used subgroup analysis and multivariate logistic regression models (Rhead *et al.*, 2007 [Chapter 6]). We found that the association of i1-type strains with gastric cancer among our Iranian patients was independent of these other pathogenicity markers. Similarly, Basso *et al*. (2008) also found using multivariate analysis that only the i1 allele was independently associated with peptic ulcer, although infection with an i1-type strain did not significantly increase gastric cancer risk when adjusted for *cagA* phosphorylation motif number. Hussein *et al*. (2008) did not find any association between gastric ulcer and other *vacA* allelic types, or the presence of *cagA*, indicating that the i1 allele was the only determinant in their study. In contrast, Ogiwara *et al*. (2009) report that using multiple linear regression analysis, no single factor was associated with gastric disease, although their univariate analysis showed that the i1 allele gave the highest odds ratio for gastric cancer, and was second to *cagA* presence for

determining gastric ulcer risk (Ogiwara *et al.*, 2009). Thus, these studies, which span a diverse range of geographical locations, support our initial observation that the *vacA* i-region is an important marker of *H. pylori*-related gastric disease.

As discussed above, the *vacA* i1 allele predominates among *H. pylori* isolates from many East Asian countries. While this may explain why such countries have a high incidence of gastric disease, it means that i-region type is less useful for determining disease risk among such populations. Sheu *et al*. (2009) analysed the i-region nucleotide sequence of 39 i1-type strains from Taiwanese patients, and found that strains with a nonsynonomous substitution encoding glycine rather than serine, asparagine or aspartic acid at the ninth residue of cluster B, were significantly associated with peptic ulcer disease and gastric cancer. More recently, Jones *et al*. (2011) studied the i-region sequences of 222 strains from South Korean patients, and found that such variation at position 9 of cluster B was only associated with disease among strains in which CagA did not have the Asian phosphorylation motif (EPIYA-ABD). The authors also described another polymorphic site, five residues down from cluster A, which was either serine or leucine: i1 sequences were predominantly serine, while i2 sequences were invariably leucine. Patients with gastric cancer were significantly more likely to be infected with a strain having serine at this position than non-cancer patients. Further studies may determine whether these polymorphic sites within the i-region are associated with disease in other populations, or have any effect on the vacuolating activity of the toxin.

7.4 Autotransport mechanism of VacA secretion

The polypeptide encoded by the *vacA* gene has a typical autotransporter organisation, containing an N-terminal signal sequence of 30-33 residues, and an ~50kDa C-terminal domain, both of which are cleaved during secretion to produce the mature toxin (Cover *et al.*, 1994; Phadnis *et al.*, 1994; Schmitt and Haas, 1994; Telford *et al.*, 1994). The cleaved C-terminal domain remains associated with the cell (Telford *et al.*, 1994), and shares homology with autotransporters from other bacterial species, including 14 putative amphipathic **13**-strands typical of outer membrane spanning domains (Loveless and Saier, 1997). Furthermore, fusing the VacA cell-associated C-terminal domain to the cholera toxin B

subunit results in the secretion of the latter to the *H. pylori* surface (Fischer *et al.*, 2001). A conserved feature of some autotransporters is the presence of a pair of cysteine residues, most commonly 11 residues apart, located towards the C-terminus of the passenger domain, and we have shown that substitution of serine for either of these cysteine residues in VacA results in severely reduced levels of toxin in culture supernatants, suggesting they are important for the secretion process (Letley *et al.*, 2006 [Chapter 5]). Several studies have suggested the existance of an autochaperone region at the C-terminal end of autotransporter passenger domains, and we proposed that mutagenesis of the cysteine residues may affect the conformation of such a region in VacA (as discussed in Letley *et al.*, 2006 [Chapter 5]).

Despite no sequence homology between autotransporter passenger domains, all the structures solved to date are, like VacA, largely composed of an extended right-handed 13-helix from which other functional domains may protrude (for a recent review see Benz and Schmidt, 2011). Translocation of the pertactin passenger domain has been shown to begin at its C-terminal end, and vectorial folding of the 13-helix as it exits the transporter domain's pore is suggested to drive the secretion process in the absence of ATP or a proton motive force across the outer membrane (Junker *et al.*, 2009). Studying the folding kinetics of purified passenger domains has revealed that the C-terminal end forms a more stable structure (Junker *et al.*, 2006; Renn and Clark, 2008), consisting of the end of the 13-helix and the autochaperone domain, which caps the end of the helix protecting its hydrophobic interior. The autochaperone domain is thought to fold on exiting the translocator pore and initiate the stable folding of the 13-helix. Mutagenesis studies of the putative autochaperone regions of IcsA (May and Morona, 2008), Hbp (Soprova *et al.*, 2010) and BrkA (Oliver *et al.*, 2003) highlight the importance of this region for passenger domain secretion. The cysteine residues at the C-terminus of VacA form a disulphide bond located within the autochaperone region, immediately behind the 13-helix cap (Gangwer *et al.*, 2007). Thus, they are ideally placed to stabilise the formation of the autochaperone region promoting folding of the rest of the passenger domain. Interestingly, deletion of a single coil near the C-terminus of the VacA 13-helix greatly reduced the amount of secreted toxin (Ivie *et al.*, 2010), similar to the effect we observed for substitution of the cysteine residues, confirming that the stability of the C-terminus of VacA is critical, as observed for other autotransporters. The IgA1 protease of

Haemophilus influenzae is another autotransporter with a cysteine pair located in the passenger domain with an 11 residue spacing, as occurs in VacA. The crystal structure of the IgA1 passenger domain reveals that these cysteines form a disulphide bond which stabilises a two strand 13-sheet in a domain which protrudes about halfway down the length of the 13-helix spine (Johnson *et al.*, 2009). This domain is suggested to form part of the substrate binding site for the protease, rather than be involved in secretion. Thus, the similarly spaced cysteine pairs observed in some autotransporters may be stabilising small domains with different roles for each protein, rather than being part of a common mechanism for secretion. Presumably, restrictions on polypeptide folding prior to translocation have placed limitations on the number of cysteines and their spacing in autotransporter passenger domains (Jong *et al.*, 2007).

The disulphide bond between the VacA cysteine pair occurs between an LI-helix and a 13-strand (Gangwer *et al.*, 2007). A favoured model of autotransporter secretion is the formation of a hairpin in the C-terminus of the passenger domain which enters the translocator pore from the periplasmic side to initiate translocation. The passenger domains of IcsA and EspP form disulphide bonds and undergo some folding in the periplasm prior to secretion (Brandon and Goldberg, 2001; Skillman *et al.*, 2005), therefore it is possible that the VacA disulphide bond similarly forms in the periplasm. Furthermore, the crystal structures of several autotransporter translocator domains reveal the presence of an LI-helix within the pore (reviewed in Benz and Schmidt, 2011), demonstrating that such a structure can be accomodated. Thus it is possible that the VacA cysteine pair stabilise the formation of a hairpin between an LI-helix and 13-strand in the periplasm which directs translocation of the C-terminus of the passenger through the pore. These structures may then assist with the folding of the rest of the autochaperone, and ultimately the 13-helix upon exiting the pore. We found that the C771S mutation had a greater impact on VacA levels than C782S, suggesting an additional effect beyond disruption of disulphide bond formation. The cysteine at position 771 resides within the 13-strand, which appears to form a small antiparallel sheet with two further 13-sheets within the autochaperone domain. It is possible that the substitution of serine at this position has a slightly greater effect on the overall structure of the autochaperone domain than at position 782 within the LI-helix.

Mapping sequence variation between both m1 and m2 sequences to the crystal structure of the VacA p55 domain revealed just two conserved, surface-exposed regions: one located at the N-terminus; and a conserved pocket formed from two long 13-helix loops and the disulphide bond at the C-terminus (Gangwer *et al.*, 2007). It was suggested that this feature may represent a receptor binding site shared between m1 and m2 forms of the toxin (Gangwer *et al.*, 2007). Thus, the C-terminal domain of VacA may be important for both autochaperone activity during secretion, and binding of the mature toxin to host cells. We found that the small amount of mutated toxin, VacA C782S, secreted in to the culture supernatant was still able to vacuolate HeLa and RK13 cells (Letley *et al.*, 2006 [Chapter 5]). Presumably, the C-terminal domain of a fraction of toxin molecules was still able to fold correctly, despite losing the stabilising effect of the disulphide bond, to allow secretion, and therefore the involvement of this region in receptor binding cannot be ruled out. An alternative role for this conserved pocket could be to interact with an accessory component required for toxin secretion. While autotransporters were originally proposed to secrete their passenger domains across the outer membrane unassisted, more recent studies have suggested the involvement of other proteins, including the BAM complex required for the insertion of 13-barrel structures into the outer membrane (reviewed in Benz and Schmidt, 2011). Cross-linking studies have shown an interaction between BamA and the EspP passenger domain during its translocation, suggesting that the BAM complex may be involved with both the insertion of the autotransporter pore domain into the outer membrane, and the passenger domain translocation itself (Ieva and Bernstein, 2009). If the conserved pocket in the VacA structure was involved in an association with the BAM complex, disruption of the disulphide bond by cysteine substitution may affect this interaction, thereby hindering translocation of the toxin.

7.5 Conclusion

For more than two decades, the vacuolating cytotoxin of *H. pylori* has been the subject of extensive study. The description of *vacA* polymorphism, and the ability to identify alleles by a simple PCR typing system has resulted in the characterisation of *vacA* diversity among clinical isolates from all regions of the world, including the African continent, which we were the first to describe. In many countries, associations between the *vacA* s1 and m1 alleles and

H. pylori-related disease have been found, making the cytotoxin one of the major virulence determinants of this important gastric pathogen. Using an isogenic mutant and hybrid approach, we characterised the effect of signal and mid region allelic differences on vacuolating activity. Our data, in combination with other studies, has shown that the *vacA* signal region determines vacuolating activity, and mid region polymorphisms affect the cell specificity of the toxin, by altering its binding to host cells. Collectively, these results demonstrate the functional importance of these two regions upon which many *vacA* typing studies have been based. We have also identified a third determinant of vacuolating activity, the intermediate region, which we showed to be the most important disease marker. Our initial characterisation of this region has generated a lot of interest, and several studies have now confirmed its significance for determining disease risk. Future studies are now needed to determine why i2-type polymorphisms reduce the vacuolating activity of the toxin, and to characterise the importance of the i-region for other effects of the toxin. Finally, in contrast to the characterisation of *vacA* polymorphisms, we have identified two cysteine residues, conserved among VacA types, which appear to play an important structural role in the secretion or stable folding of the toxin. These residues reside within an interesting region of the p55 structure, in close proximity to the 13-helix cap, and a conserved pocket on the surface of the protein, and our results will add to the future characterisation of these potentially important structures.

CHAPTER 8
REFERENCES

Adamsky, K., Arnold, K., Sabanay, H., and Peles, E. (2003) Junctional protein MAGI-3 interacts with receptor tyrosine phosphatase 0 (RPTP(3) and tyrosine-phosphorylated proteins. *Journal of Cell Science* 116: 1279-1289.

Adrian, M., Cover, T.L., Dubochet, J., and Heuser, J.E. (2002) Multiple oligomeric states of the *Helicobacter pylori* vacuolating toxin demonstrated by cryo-electron microscopy. *Journal of Molecular Biology* 318: 121-133.

Agha, A., and Graham, D.Y. (2005) Evidence-based examination of the African enigma in relation to *Helicobacter pylori* infection. *Scandinavian Journal of Gastroenterology* 40: 523-529.

Akada, J.K., Aoki, H., Torigoe, Y., Kitagawa, T., Kurazono, H., Hoshida, H., Nishikawa, J., Terai, S., Matsuzaki, M., Hirayama, T., Nakazawa, T., Akada, R., and Nakamura, K. (2010) *Helicobacter pylori* CagA inhibits endocytosis of cytotoxin VacA in host cells. *Disease Models and Mechanisms* 3: 605-617.

Akanuma, M., Maeda, S., Ogura, K., Mitsuno, Y., Hirata, Y., Ikenoue, T., Otsuka, M., Watanabe, T., Yamaji, Y., Yoshida, H., Kawabe, T., Shiratori, Y., and Omata, M. (2002) The evaluation of putative virulence factors of *Helicobacter pylori* for gastroduodenal disease by use of a short-term Mongolian gerbil infection model. *Journal of Infectious Diseases* 185: 341-347.

Akopyants, N.S., Clifton, S.W., Kersulyte, D., Crabtree, J.E., Youree, B.E., Reece, C.A., Bukanov, N.O., Drazek, E.S., Roe, B.A., and Berg, D.E. (1998) Analyses of the *cag* pathogenicity island of *Helicobacter pylori*. *Molecular Microbiology* 28: 37-53.

Algood, H.M., Torres, V.J., Unutmaz, D., and Cover, T.L. (2007) Resistance of primary murine CD4+ T cells to *Helicobacter pylori* vacuolating cytotoxin. *Infection and Immunity* 75: 334-341.

Amado, M., Carneiro, F., Seixas, M., Clausen, H., and Sobrinho-Simoes, M. (1998) Dimeric sialyl-Le(x) expression in gastric carcinoma correlates with venous invasion and poor outcome. *Gastroenterology* 114: 462-470.

Amieva, M.R., Vogelmann, R., Covacci, A., Tompkins, L.S., Nelson, W.J., and Falkow, S. (2003) Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science* 300: 1430-1434.

Ando, T., Peek, R.M., Jr., Lee, Y.C., Krishna, U., Kusugami, K., and Blaser, M.J. (2002a) Host cell responses to genotypically similar *Helicobacter pylori* isolates from United States and Japan. *Clinical and Diagnostic Laboratory Immunology* 9: 167-175.

Ando, T., Peek, R.M., Pride, D., Levine, S.M., Takata, T., Lee, Y.C., Kusugami, K., van der Ende, A., Kuipers, E.J., Kusters, J.G., and Blaser, M.J. (2002b) Polymorphisms of *Helicobacter pylori* HP0638 reflect geographic origin and correlate with *cagA* status. *Journal of Clinical Microbiology* 40: 239-246.

Antonin, W., Holroyd, C., Fasshauer, D., Pabst, S., Von Mollard, G.F., and Jahn, R. (2000) A SNARE complex mediating fusion of late endosomes defines conserved properties of SNARE structure and function. *EMBO Journal* 19: 6453-6464.

Appelmelk, B.J., Simoons-Smit, I., Negrini, R., Moran, A.P., Aspinall, G.O., Forte, J.G., De Vries, T., Quan, H., Verboom, T., Maaskant, J.J., Ghiara, P., Kuipers, E.J., Bloemena, E., Tadema, T.M., Townsend, R.R., Tyagarajan, K., Crothers, J.M., Jr., Monteiro, M.A., Savio, A., and De Graaff, J. (1996) Potential role of molecular mimicry between *Helicobacter pylori* lipopolysaccharide and host Lewis blood group antigens in autoimmunity. *Infection and Immunity* **64**: 2031-2040.

Arachchi, H.S., Kalra, V., Lal, B., Bhatia, V., Baba, C.S., Chakravarthy, S., Rohatgi, S., Sarma, P.M., Mishra, V., Das, B., and Ahuja, V. (2007) Prevalence of duodenal ulcer-promoting gene (*dupA*) of *Helicobacter pylori* in patients with duodenal ulcer in North Indian population. *Helicobacter* **12**: 591-597.

Argent, R.H., Kidd, M., Owen, R.J., Thomas, R.J., Limb, M.C., and Atherton, J.C. (2004a) Determinants and consequences of different levels of CagA phosphorylation for clinical isolates of *Helicobacter pylori*. *Gastroenterology* **127**: 514-523.

Argent, R.H., McGarr, C., and Atherton, J.C. (2004b) Brefeldin A enhances *Helicobacter pylori* vacuolating cytotoxin-induced vacuolation of epithelial cells. *FEMS Microbiology Letters* **237**: 163-170.

Argent, R.H., Burette, A., Miendje Deyi, V.Y., and Atherton, J.C. (2007) The presence of *dupA* in *Helicobacter pylori* is not significantly associated with duodenal ulceration in Belgium, South Africa, China, or North America. *Clinical Infectious Diseases* **45**: 1204-1206.

Argent, R.H., Thomas, R.J., Letley, D.P., Rittig, M.G., Hardie, K.R., and Atherton, J.C. (2008) Functional association between the *Helicobacter pylori* virulence factors VacA and CagA. *Journal of Medical Microbiology* **57**: 145-150.

Asahi, M., Azuma, T., Ito, S., Ito, Y., Suto, H., Nagai, Y., Tsubokawa, M., Tohyama, Y., Maeda, S., Omata, M., Suzuki, T., and Sasakawa, C. (2000) *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. *Journal of Experimental Medicine* **191**: 593-602.

Asahi, M., Tanaka, Y., Izumi, T., Ito, Y., Naiki, H., Kersulyte, D., Tsujikawa, K., Saito, M., Sada, K., Yanagi, S., Fujikawa, A., Noda, M., and Itokawa, Y. (2003) *Helicobacter pylori* CagA containing ITAM-like sequences localized to lipid rafts negatively regulates VacA-induced signaling in vivo. *Helicobacter* **8**: 1-14.

Aspholm, M., Olfat, F.O., Norden, J., Sonden, B., Lundberg, C., Sjostrom, R., Altraja, S., Odenbreit, S., Haas, R., Wadstrom, T., Engstrand, L., Semino-Mora, C., Liu, H., Dubois, A., Teneberg, S., Arnqvist, A., and Boren, T. (2006) SabA is the *H. pylori* hemagglutinin and is polymorphic in binding to sialylated glycans. *PLoS Pathogens* **2**: e110.

Aspinall, G.O., and Monteiro, M.A. (1996) Lipopolysaccharides of Helicobacter pylori strains P466 and MO19: structures of the O antigen and core oligosaccharide regions. *Biochemistry* **35**: 2498-2504.

Aspinall, G.O., Monteiro, M.A., Pang, H., Walsh, E.J., and Moran, A.P. (1996) Lipopolysaccharide of the *Helicobacter pylori* type strain NCTC 11637 (ATCC 43504):

structure of the O antigen chain and core oligosaccharide regions. *Biochemistry* **35**: 2489-2497.

Asrat, D., Nilsson, I., Mengistu, Y., Kassa, E., Ashenafi, S., Ayenew, K., Wadstrom, T., and Abu-Al-Soud, W. (2004) Prevalence of *Helicobacter pylori vacA* and *cagA* genotypes in Ethiopian dyspeptic patients. *Journal of Clinical Microbiology* **42**: 2682-2684.

Atherton, J.C., Cao, P., Peek, R.M., Tummuru, M.K.R., Blaser, M.J., and Cover, T.L. (1995) Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*: Association of specific *vacA* types with cytotoxin production and peptic ulceration. *Journal of Biological Chemistry* **270**: 17771-17777.

Atherton, J.C., Peek Jr, R.M., Tham, K.T., Cover, T.L., and Blaser, M.J. (1997) Clinical and pathological importance of heterogeneity in *vacA*, the vacuolating cytotoxin gene of *Helicobacter pylori*. *Gastroenterology* **112**: 92-99.

Atherton, J.C., Cover, T.L., Twells, R.J., Morales, M.R., Hawkey, C.J., and Blaser, M.J. (1999a) Simple and accurate PCR-based system for typing vacuolating cytotoxin alleles of *Helicobacter pylori*. *Journal of Clinical Microbiology* **37**: 2979-2982.

Atherton, J.C., Sharp, P.M., Cover, T.L., Gonzalez-Valencia, G., Peek Jr, R.M., Thompson, S.A., Hawkey, C.J., and Blaser, M.J. (1999b) Vacuolating cytotoxin (*vacA*) alleles of *Helicobacter pylori* comprise two geographically widespread types, m1 and m2, and have evolved through limited recombination. *Current Microbiology* **39**: 211-218.

Ayala, G., Torres, L., Espinosa, M., Fierros-Zarate, G., Maldonado, V., and Meléndez-Zajgla, J. (2006) External membrane vesicles from *Helicobacter pylori* induce apoptosis in gastric epithelial cells. *FEMS Microbiology Letters* **260**: 178-185.

Azuma, T., Yamakawa, A., Yamazaki, S., Fukuta, K., Ohtani, M., Ito, Y., Dojo, M., Yamazaki, Y., and Kuriyama, M. (2002) Correlation between variation of the 3' region of the *cagA* gene in *Helicobacter pylori* and disease outcome in Japan. *Journal of Infectious Diseases* **186**: 1621-1630.

Backert, S., Ziska, E., Brinkmann, V., Zimny-Arndt, U., Fauconnier, A., Jungblut, P.R., Naumann, M., and Meyer, T.F. (2000) Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. *Cellular Microbiology* **2**: 155-164.

Backstrom, A., Lundberg, C., Kersulyte, D., Berg, D.E., Boren, T., and Arnqvist, A. (2004) Metastability of *Helicobacter pylori bab* adhesin genes and dynamics in Lewis b antigen binding. *Proceedings of the National Academy of Sciences (U.S.A.)* **101**: 16923-16928.

Bagnoli, F., Buti, L., Tompkins, L., Covacci, A., and Amieva, M.R. (2005) *Helicobacter pylori* CagA induces a transition from polarized to invasive phenotypes in MDCK cells. *Proceedings of the National Academy of Sciences (U.S.A.)* **102**: 16339-16344.

Basso, D., Navaglia, F., Brigato, L., Piva, M.G., Toma, A., Greco, E., Di Mario, F., Galeotti, F., Roveroni, G., Corsini, A., and Plebani, M. (1998) Analysis of *Helicobacter pylori vacA* and *cagA* genotypes and serum antibody profile in benign and malignant gastroduodenal diseases. *Gut* **43**: 182-186.

Basso, D., Zambon, C.F., Letley, D.P., Stranges, A., Marchet, A., Rhead, J.L., Schiavon, S., Guariso, G., Ceroti, M., Nitti, D., Rugge, M., Plebani, M., and Atherton, J.C. (2008) Clinical relevance of *Helicobacter pylori cagA* and *vacA* gene polymorphisms. *Gastroenterology*: DOI: 10.1053/j.gastro.2008.1003.1041.

Bebb, J.R., Letley, D.P., Thomas, R.J., Aviles, F., Collins, H.M., Watson, S.A., Hand, N.M., Zaitoun, A., and Atherton, J.C. (2003) *Helicobacter pylori* upregulates matrilysin (MMP-7) in epithelial cells *in vivo* and *in vitro* in a Cag dependent manner. *Gut* **52**: 1408-1413.

Ben Mansour, K., Fendri, C., Zribi, M., Masmoudi, A., Labbene, M., Fillali, A., Ben Mami, N., Najjar, T., Meherzi, A., Sfar, T., and Burucoa, C. (2010) Prevalence of *Helicobacter pylori vacA*, *cagA*, *iceA* and *oipA* genotypes in Tunisian patients. *Annals of Clinical Microbiology and Antimicrobials* **9**: 10-16.

Benz, I., and Schmidt, M.A. (2011) Structures and functions of autotransporter proteins in microbial pathogens. *International Journal of Medical Microbiology*: doi:10.1016/j.ijmm.2011.1003.1003.

Bizzozero, G. (1893) Ueber die schlauchförmigen Drüsen des Magendarmkanals und die Beziehungen ihres Epithels zu dem Oberflächenepithel der Schleimhaut. *Arch Mikr Anat* **42**: 82-152.

Bode, G., Malfertheiner, P., Lehnhardt, G., Nilius, M., and Ditschuneit, H. (1993) Ultrastructural localization of urease of *Helicobacter pylori*. *Medical Microbiology and Immunology* **182**: 233-242.

Boncristiano, M., Paccani, S.R., Barone, S., Ulivieri, C., Patrussi, L., Ilver, D., Amedei, A., D'Elios, M.M., Telford, J.L., and Baldari, C.T. (2003) The *Helicobacter pylori* vacuolating toxin inhibits T cell activation by two independent mechanisms. *Journal of Experimental Medicine* **198**: 1887-1897.

Botham, C.M., Wandler, A.M., and Guillemin, K. (2008) A transgenic Drosophila model demonstrates that the *Helicobacter pylori* CagA protein functions as a eukaryotic Gab adaptor. *PLoS Pathogens* **4**: e1000064.

Brandon, L.D., and Goldberg, M.B. (2001) Periplasmic transit and disulphide bond formation of the autotransported *Shigella* protein IcsA. *Journal of Bacteriology* **183**: 951-958.

Brandt, S., Kwok, T., Hartig, R., Konig, W., and Backert, S. (2005) NF-kappaB activation and potentiation of proinflammatory responses by the *Helicobacter pylori* CagA protein. *Proceedings of the National Academy of Sciences (U.S.A.)* **102**: 9300-9305.

Bumann, D., Aksu, S., Wendland, M., Janek, K., Zimny-Arndt, U., Sabarth, N., Meyer, T.F., and Jungblut, P.R. (2002) Proteome analysis of secreted proteins of the gastric pathogen *Helicobacter pylori*. *Infection and Immunity* **70**: 3396-3403.

Burroni, D., Lupetti, P., Pagliaccia, C., Reyrat, J.M., Dallai, R., Rappuoli, R., and Telford, J.L. (1998) Deletion of the major proteolytic site of the *Helicobacter pylori* cytotoxin does not influence toxin activity but favours assembly of the toxin into hexameric structures. *Infection and Immunity* **66**: 5547-5550.

Bury-Mone, S., Skouloubris, S., Dauga, C., Thiberge, J.M., Dailidiene, D., Berg, D.E., Labigne, A., and De Reuse, H. (2003) Presence of active aliphatic amidases in *Helicobacter* species able to colonize the stomach. *Infection and Immunity* **71**: 5613-5622.

Caputo, R., Tuccillo, C., Manzo, B.A., Zarrilli, R., Tortora, G., Del Vecchio Blanco, C., Ricci, V., Ciardiello, F., and Romano, M. (2003) *Helicobacter pylori* VacA toxin up-regulates vascular endothelial growth factor expression in MKN 28 gastric cells through an epidermal growth factor receptor-, cyclooxygenase-2-dependent mechanism. *Clinical Cancer Research* **9**: 2015-2021.

Catalano, M., Matteo, M., Barbolla, R.E., Jimenez Vega, D.E., Crespo, O., Leanza, A.G., Toppor, J., and Antelo, P. (2001) *Helicobacter pylori vacA* genotypes, *cagA* status and *ureA-B* polymorphism in isolates recovered from an Argentine population. *Diagnostic Microbiology and Infectious Disease* **41**: 205-210.

Cavanna, L., Pagani, R., Seghini, P., Zangrandi, A., and Paties, C. (2008) High grade B-cell gastric lymphoma with complete pathologic remission after eradication of *Helicobacter pylori infection*: report of a case and review of the literature. *World Journal of Surgical Oncology* **6**: 35-41.

Censini, S., Lange, C., Xiang, Z., Crabtree, J.E., Ghiara, P., Borodovsky, M., Rappuoli, R., and Covacci, A. (1996) *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proceedings of the National Academy of Sciences (U.S.A.)* **93**: 14648-14653.

Chen, L.T., Lin, J.T., Tai, J.J., Chen, G.H., Yeh, H.Z., Yang, S.S., Wang, H.P., Kuo, S.H., Sheu, B.S., Jan, C.M., Wang, W.M., Wang, T.E., Wu, C.W., Chen, C.L., Su, I.J., Whang-Peng, J., and Cheng, A.L. (2005) Long-term results of anti-*Helicobacter pylori* therapy in early-stage gastric high-grade transformed MALT lymphoma. *Journal of the National Cancer Institute* **97**: 1345-1353.

Cho, S.J., Kang, N.S., Park, S.Y., Kim, B.O., Rhee, D.K., and Pyo, S. (2003) Induction of apoptosis and expression of apoptosis related genes in human epithelial carcinoma cells by *Helicobacter pylori* VacA toxin. *Toxicon* **42**: 601-611.

Chung, C., Olivares, A., Torres, E., Yilmaz, O., Cohen, H., and Perez-Perez, G. (2010) Diversity of VacA intermediate region among *Helicobacter pylori* strains from several regions of the world. *Journal of Clinical Microbiology* **48**: 690-696.

Churin, Y., Al-Ghoul, L., Kepp, O., Meyer, T.F., Birchmeier, W., and Naumann, M. (2003) *Helicobacter pylori* CagA protein targets the c-Met receptor and enhances the motogenic response. *Journal of Cell Biology* **161**: 249-255.

Clyne, M., Dolan, B., and Reeves, E.P. (2007) Bacterial factors that mediate colonization of the stomach and virulence of *Helicobacter pylori*. *FEMS Microbiology Letters* **268**: 135143.

Con, S.A., Takeuchi, H., Valerin, A.L., Con-Wong, R., Con-Chin, G.R., Con-Chin, V.G., Nishioka, M., Mena, F., Brenes, F., Yasuda, N., Araki, K., and Sugiura, T. (2007)

Diversity of *Helicobacter pylori cagA* and *vacA* genes in Costa Rica: its relationship with atrophic gastritis and gastric cancer. *Helicobacter* **12**: 547-552.

Correa, P. (1988) A human model of gastric carcinogenesis. *Cancer Research* **48**: 3554-3560.

Cover, T.L., Dooley, C.P., and Blaser, M.J. (1990) Characterisation of and human serologic response to proteins in *Helicobacter pylori* broth culture supernatants with vacuolising cytotoxin activity. *Infection and Immunity* **58**: 603-610.

Cover, T.L., and Blaser, M.J. (1992) Purification and characterisation of the vacuolating toxin from *Helicobacter pylori*. *Journal of Biological Chemistry* **267**: 10570-10575.

Cover, T.L., Cao, P., Murthy, U.K., Sipple, M.S., and Blaser, M.J. (1992a) Serum neutralising antibody response to the vacuolating cytotoxin of *Helicobacter pylori*. *The Journal of Clinical Investigation* **90**: 913-918.

Cover, T.L., Halter, S.A., and Blaser, M.J. (1992b) Characterisation of HeLa cell vacuoles induced by *Helicobacter pylori* broth culture supernatant. *Human Pathology* **23**: 1004-1010.

Cover, T.L., Vaughn, S.G., Cao, P., and Blaser, M.J. (1992c) Potentiation of *Helicobacter pylori* vacuolating toxin activity by nicotine and other weak bases. *Journal of Infectious Diseases* **166**: 1073-1078.

Cover, T.L., Reddy, L.Y., and Blaser, M.J. (1993) Effects of ATPase inhibitors on the response of HeLa cells to *Helicobacter pylori* vacuolating toxin. *Infection and Immunity* **61**: 1427-1431.

Cover, T.L., Tummuru, M.K.R., Cao, P., Thompson, S.A., and Blaser, M.J. (1994) Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. *Journal of Biological Chemistry* **269**: 10566-10573.

Cover, T.L., Hanson, P.I., and Heuser, J.E. (1997) Acid-induced dissociation of VacA, the Helicobacter vacuolating cytotoxin, reveals its pattern of assembly. *Journal of Cell Biology* **138**: 759-769.

Cover, T.L., Krishna, U.S., Israel, D.A., and Peek, R.M., Jr. (2003) Induction of gastric epithelial cell apoptosis by *Helicobacter pylori* vacuolating cytotoxin. *Cancer Research* **63**: 951-957.

Croxen, M.A., Sisson, G., Melano, R., and Hoffman, P.S. (2006) The *Helicobacter pylori* chemotaxis receptor TlpB (HP0103) is required for pH taxis and for colonization of the gastric mucosa. *Journal of Bacteriology* **188**: 2656-2665.

Czajkowsky, D.M., Iwamoto, H., Cover, T.L., and Shao, Z. (1999) The vacuolating toxin from *Helicobacter pylori* forms hexameric pores in lipid bilayers at low pH. *Proceedings of the National Academy of Sciences (U.S.A.)* **96**: 2001-2006.

Czajkowsky, D.M., Iwamoto, H., Szabo, G., Cover, T.L., and Shao, Z. (2005) Mimicry of a host anion channel by a *Helicobacter pylori* pore-forming toxin. *Biophysical Journal* **89**: 3093-3101.

de Bernard, M., Papini, E., de Filippis, V., Gottardi, E., Telford, J., Manetti, R., Fontana, A., Rappuoli, R., and Montecucco, C. (1995) Low pH activates the vacuolating toxin of

Helicobacter pylori, **which becomes acid and pepsin resistant.** *Journal of Biological Chemistry* ***270*****: 23937-23940.**

de Bernard, M., Burroni, D., Papini, E., Rappuoli, R., Telford, J., and Montecucco, C. (1998a) Identification of the *Helicobacter pylori* **VacA toxin domain active in the cell cytosol.** *Infection and Immunity* ***66*****: 6014-6016.**

de Bernard, M., Moschioni, M., Papini, E., Telford, J.L., Rappuoli, R., and Montecucco, C. (1998b) TPA and butyrate increase cell sensitivity to the vacuolating toxin of *Helicobacter pylori*. *FEBS Letters* ***436*****: 218-222.**

de Bernard, M., Moschioni, M., Habermann, A., Griffiths, G., and Montecucco, C. (2002) Cell vacuolization induced by *Helicobacter pylori* **VacA cytotoxin does not depend on late endosomal SNAREs.** *Cellular Microbiology* ***4*****: 11-18.**

de Bernard, M., Cappon, A., Pancotto, L., Ruggiero, P., Rivera, J., Del Giudice, G., and Montecucco, C. (2005) The *Helicobacter pylori* **VacA cytotoxin activates RBL-2H3 cells by inducing cytosolic calcium oscillations.** *Cellular Microbiology* ***7*****: 191-198.**

De Francesco, V., Margiotta, M., Zullo, A., Hassan, C., Giorgio, F., Zotti, M., Stoppino, G., Bastianelli, A., Diterlizzi, F., Verderosa, G., Morini, S., Panella, C., and Ierardi, E. (2009) *Helicobacter pylori vacA* **arrangement and related diseases: a retrospective study over a period of 15 years.** *Digestive Diseases and Sciences* ***54*****: 97-102.**

De Guzman, B.B., Hisatsune, J., Nakayama, M., Yahiro, K., Wada, A., Yamasaki, E., Nishi, Y., Yamazaki, S., Azuma, T., Ito, Y., Ohtani, M., van der Wijk, T., den Hertog, J., Moss, J., and Hirayama, T. (2005) Cytotoxicity and recognition of receptor-like protein tyrosine phosphatases, RPTPa and RPTPb, by *Helicobacter pylori* **m2VacA.** *Cellular Microbiology* ***7*****: 1285-1293.**

de Jonge, R., Durrani, Z., Rijpkema, S.G., Kuipers, E.J., van Vliet, A.H., and Kusters, J.G. (2004a) Role of the *Helicobacter pylori* **outer-membrane proteins AlpA and AlpB in colonization of the guinea pig stomach.** *Journal of Medical Microbiology* ***53*****: 375-379.**

de Jonge, R., Pot, R.G., Loffeld, R.J., van Vliet, A.H., Kuipers, E.J., and Kusters, J.G. (2004b) The functional status of the *Helicobacter pylori sabB* **adhesin gene as a putative marker for disease outcome.** *Helicobacter* ***9*****: 158-164.**

de Sablet, T., Piazuelo, M.B., Shaffer, C.L., Schneider, B.G., Asim, M., Chaturvedi, R., Bravo, L.E., Sicinschi, L.A., Delgado, A.G., Mera, R.M., Israel, D.A., Romero-Gallo, J., Peek, R.M., Jr., Cover, T.L., Correa, P., and Wilson, K.T. (2011) Phylogeographic origin of *Helicobacter pylori* **is a determinant of gastric cancer risk.** *Gut*: **doi:10.1136/gut.2010.234468.**

Diaz, M.I., Valdivia, A., Martinez, P., Palacios, J.L., Harris, P., Novales, J., Garrido, E., Valderrama, D., Shilling, C., Kirberg, A., Hebel, E., Fierro, J., Bravo, R., Siegel, F., Leon, G., Klapp, G., and Venegas, A. (2005) *Helicobacter pylori vacA* **s1a and s1b alleles from clinical isolates from different regions of Chile show a distinct geographic distribution.** *World Journal of Gastroenterology* ***11*****: 6366-6372.**

Domanska, G., Motz, C., Meinecke, M., Harsman, A., Papatheodorou, P., Reljic, B., Dian-Lothrop, E.A., Galmiche, A., Kepp, O., Becker, L., Gunnewig, K., Wagner, R., and Rassow, J. (2010) *Helicobacter pylori* VacA toxin/subunit p34: targeting of an anion channel to the inner mitochondrial membrane. *PLoS Pathogens* 6: e1000878.

Donati, M., Moreno, S., Storni, E., Tucci, A., Poli, L., Mazzoni, C., Varoli, O., Sambri, V., Farencena, A., and Cevenini, R. (1997) Detection of serum antibodies to CagA and VacA and of serum neutralizing activity for vacuolating cytotoxin in patients with *Helicobacter pylori*-induced gastritis. *Clinical and Diagnostic Laboratory Immunology* 4: 478-482.

Dossumbekova, A., Prinz, C., Mages, J., Lang, R., Kusters, J.G., Van Vliet, A.H., Reindl, W., Backert, S., Saur, D., Schmid, R.M., and Rad, R. (2006) *Helicobacter pylori* HopH (OipA) and bacterial pathogenicity: genetic and functional genomic analysis of *hopH* gene polymorphisms. *Journal of Infectious Diseases* 194: 1346-1355.

Douraghi, M., Mohammadi, M., Oghalaie, A., Abdirad, A., Mohagheghi, M.A., Hosseini, M.E., Zeraati, H., Ghasemi, A., Esmaieli, M., and Mohajerani, N. (2008) *dupA* as a risk determinant in *Helicobacter pylori* infection. *Journal of Medical Microbiology* 57: 554-562.

Dunn, B.E., Vakil, N.B., Schneider, B.G., Miller, M.M., Zitzer, J.B., Peutz, T., and Phadnis, S.H. (1997) Localization of *Helicobacter pylori* urease and heat shock protein in human gastric biopsies. *Infection and Immunity* 65: 1181-1188.

Eaton, K.A., Brooks, C.L., Morgan, D.R., and Krakowka, S. (1991) Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infection and Immunity* 59: 2470-2475.

Eaton, K.A., Morgan, D.R., and Krakowka, S. (1992) Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*. *Journal of Medical Microbiology* 37: 123-127.

Eaton, K.A., Cover, T.L., Tummuru, M.K., Blaser, M.J., and Krakowka, S. (1997) Role of vacuolating cytotoxin in gastritis due to *Helicobacter pylori* in gnotobiotic piglets. *Infection and Immunity* 65: 3462-3464.

Egan, B.J., Katicic, M., O'Connor, H.J., and O'Morain, C.A. (2007) Treatment of *Helicobacter pylori*. *Helicobacter* 12 Suppl 1: 31-37.

Eidt, S., and Stolte, M. (1993) Prevalence of lymphoid follicles and aggregates in *Helicobacter pylori* gastritis in antral and body mucosa. *Journal of Clinical Pathology* 46: 832-835.

El Mahadi, A., Young, K., Patchett, S., Domizio, P., Fedail, S., Kumar, P., and Farthing, M. (1999) Diversity in the *vacA* gene of *Helicobacter pylori* strains from Africa. *Gut* 44: A85.

El-Bez, C., Adrian, M., Dubochet, J., and Cover, T.L. (2005) High resolution structural analysis of *Helicobacter pylori* VacA toxin oligomers by cryo-negative staining electron microscopy. *Journal of Structural Biology* 151: 215-228.

Evans, D.G., Queiroz, D.M., Mendes, E.N., and Evans Jr, D.J. (1998) *Helicobacter pylori cagA* status and s and m alleles of *vacA* in isolates from individuals with a variety of *H. pylori*-associated gastric diseases. *Journal of Clinical Microbiology* 36: 3435-3437.

Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C., and Parkin, D.M. (2010) GLOBOCAN 2008, cancer incidence and mortality worldwide: IARC CancerBase No. 10 [Internet]. Lyon, France. International Agency for Research on Cancer. Available from: http://globocan.iarc.fr.

Figueiredo, C., van Doorn, L.J., Nogueira, C., Soares, J.M., Pinho, C., Figueira, P., Quint, W.G., and Carneiro, F. (2001) *Helicobacter pylori* genotypes are associated with clinical outcome in Portuguese patients and show a high prevalence of infections with multiple strains. *Scandinavian Journal of Gastroenterology* **36**: 128-135.

Figura, N., Guglielmetti, P., Rossolini, A., Barberi, A., Cusi, G., Musmanno, R.A., Russi, M., and Quaranta, S. (1989) Cytotoxin production by *Campylobacter pylori* strains isolated from patients with peptic ulcers and from patients with chronic gastritis only. *Journal of Clinical Microbiology* **27**: 225-226.

Fiocca, R., Necchi, V., Sommi, P., Ricci, V., Telford, J., Cover, T.L., and Solcia, E. (1999) Release of *Helicobacter pylori* vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and veiscles by gastric epithelium. *Journal of Pathology* **188**: 220-226.

Fischer, W., Buhrdorf, R., Gerland, E., and Haas, R. (2001) Outer membrane targeting of passenger proteins by the vacuolating cytotoxin autotransporter of *Helicobacter pylori*. *Infection and Immunity* **69**: 6769-6775.

Fitchen, N., O'Shea, P., Williams, P., and Hardie, K.R. (2003) Electrostatic sensor for identifying interactions between peptides and bacterial membranes. *Molecular Immunology* **40**: 407-411.

Fitchen, N., Letley, D.P., O'Shea, P., Atherton, J.C., Williams, P., and Hardie, K.R. (2005) All subtypes of the cytotoxin VacA adsorb to the surface of *Helicobacter pylori* post-secretion. *Journal of Medical Microbiology* **54**: 621-630.

Foo, J.H., Culvenor, J.G., Ferrero, R.L., Kwok, T., Lithgow, T., and Gabriel, K. (2010) Both the p33 and p55 subunits of the *Helicobacter pylori* VacA toxin are targeted to mammalian mitochondria. *Journal of Molecular Biology* **401**: 792-798.

Forsyth, M.H., Atherton, J.C., Blaser, M.J., and Cover, T.L. (1998) Heterogeneity in levels of vacuolating cytotoxin gene (*vacA*) transcription among *Helicobacter pylori* strains. *Infection and Immunity* **66**: 3088-3094.

Fox, J.G., Beck, P., Dangler, C.A., Whary, M.T., Wang, T.C., Shi, H.N., and Nagler-Anderson, C. (2000) Concurrent enteric helminth infection modulates inflammation and gastric immune responses and reduces *Helicobacter*-induced gastric atrophy. *Nature Medicine* **6**: 536-542.

Fox, J.G., and Wang, T.C. (2007) Inflammation, atrophy, and gastric cancer. *Journal of Clinical Investigation* **117**: 60-69.

Foynes, S., Dorrell, N., Ward, S.J., Stabler, R.A., McColm, A.A., Rycroft, A.N., and Wren, B.W. (2000) *Helicobacter pylori* possesses two CheY response regulators and a histidine

kinase sensor, CheA, which are essential for chemotaxis and colonization of the gastric mucosa. *Infection and Immunity* **68**: 2016-2023.

Franco, A.T., Johnston, E., Krishna, U., Yamaoka, Y., Israel, D.A., Nagy, T.A., Wroblewski, L.E., Piazuelo, M.B., Correa, P., and Peek, R.M., Jr. (2008) Regulation of gastric carcinogenesis by *Helicobacter pylori* virulence factors. *Cancer Research* **68**: 379-387.

Fujikawa, A., Shirasaka, D., Yamamoto, S., Ota, H., Yahiro, K., Fukada, M., Shintani, T., Wada, A., Aoyama, N., Hirayama, T., Fukamachi, H., and Noda, M. (2003) Mice deficient in protein tyrosine phosphatase receptor type Z are resistant to gastric ulcer induction by VacA of *Helicobacter pylori*. *Nature Genetics* **33**: 375-381.

Fujimura, T., Ohta, T., Oyama, K., Miyashita, T., and Miwa, K. (2006) Role of cyclooxygenase-2 in the carcinogenesis of gastrointestinal tract cancers: a review and report of personal experience. *World Journal of Gastroenterology* **12**: 1336-1345.

Galmiche, A., Rassow, J., Doye, A., Cagnol, S., Chambard, J.-C., Contamin, S., de Thillot, V., Just, I., Ricci, V., Solcia, E., Obberghen, E.V., and Boquet, P. (2000) The N-terminal 34kDa fragment of *Helicobacter pylori* vacuolating cytotoxin targets mitochondria and induces cytochrome *c* release. *EMBO Journal* **19**: 6361-6370.

Gangwer, K.A., Mushrush, D.J., Stauff, D.L., Spiller, B., McClain, M.S., Cover, T.L., and Lacy, D.B. (2007) Crystal structure of the *Helicobacter pylori* vacuolating toxin p55 domain. *Proceedings of the National Academy of Sciences (U.S.A.)* **104**: 16293-16298.

Garner, J.A., and Cover, T.L. (1995) Analysis of genetic diversity in cytotoxin-producing and non-cytotoxin-producing *Helicobacter pylori* strains. *Journal of Infectious Diseases* **172**: 290-293.

Garner, J.A., and Cover, T.L. (1996) Binding and internalization of the *Helicobacter pylori* vacuolating cytotoxin by epithelial cells. *Infection and Immunity* **64**: 4197-4203.

Garza-Gonzalez, E., Bosques-Padilla, F.J., Tijerina-Menchaca, R., and Perez-Perez, G.I. (2004) Characterisation of *Helicobacter pylori* isolates from the north-eastern region of Mexico. *Clinical Microbiology and Infection* **10**: 41-45.

Gauthier, N.C., Ricci, V., Gounon, P., Doye, A., Tauc, M., Poujeol, P., and Boquet, P. (2004) Glycosylphosphatidylinositol-anchored proteins and actin cytoskeleton modulate chloride transport by channels formed by the *Helicobacter pylori* vacuolating cytotoxin VacA in HeLa cells. *Journal of Biological Chemistry* **279**: 9481-9489.

Gauthier, N.C., Monzo, P., Kaddai, V., Doye, A., Ricci, V., and Boquet, P. (2005) *Helicobacter pylori* VacA cytotoxin: a probe for a clathrin-independent and Cdc42-dependent pinocytic pathway routed to late endosomes. *Molecular Biology of the Cell* **16**: 48524866.

Gauthier, N.C., Monzo, P., Gonzalez, T., Doye, A., Oldani, A., Gounon, P., Ricci, V., Cormont, M., and Boquet, P. (2007) Early endosomes associated with dynamic F-actin structures are required for late trafficking of *H. pylori* VacA toxin. *Journal of Cell Biology* **177**: 343354.

Gebert, B., Fischer, W., Weiss, E., Hoffmann, R., and Haas, R. (2003) *Helicobacter*

pylori vacuolating cytotoxin inhibits T lymphocyte activation. *Science* ***301***: 1099-1102.

Geisse, N.A., Cover, T.L., Henderson, R.M., and Edwardson, J.M. (2004) Targeting of *Helicobacter pylori* vacuolating toxin to lipid raft membrane domains analysed by atomic force microscopy. *Biochemical Journal* ***381***: 911-917.

Genisset, C., Galeotti, C.L., Lupetti, P., Mercati, D., Skibinski, D.A.G., Barone, S., Battistutta, R., de Bernard, M., and Telford, J.L. (2006) A *Helicobacter pylori* vacuolating toxin mutant that fails to oligomerize has a dominant negative phenotype. *Infection and Immunity* ***74***: 1786-1794.

Genisset, C., Puhar, A., Calore, F., de Bernard, M., Dell'Antone, P., and Montecucco, C. (2007) The concerted action of the *Helicobacter pylori* cytotoxin VacA and of the v-ATPase proton pump induces swelling of isolated endosomes. *Cellular Microbiology* ***9***: 1481-1490.

Genta, R.M., Hamner, H.W., and Graham, D.Y. (1993) Gastric lymphoid follicles in *Helicobacter pylori* infection: frequency, distribution, and response to triple therapy. *Human Pathology* ***24***: 577-583.

Gerhard, M., Lehn, N., Neumayer, N., Borén, T., Rad, R., Schepp, W., Miehlke, S., Classen, M., and Prinz, C. (1999) Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin. *Proceedings of the National Academy of Sciences (U.S.A.)* ***96***: 12778-12783.

Gerhard, M., Schmees, C., Voland, P., Endres, N., Sander, M., Reindl, W., Rad, R., Oelsner, M., Decker, T., Mempel, M., Hengst, L., and Prinz, C. (2005) A secreted low-molecular-weight protein from *Helicobacter pylori* induces cell-cycle arrest of T cells. *Gastroenterology* ***128***: 1327-1339.

Ghose, C., Perez-Perez, G.I., Torres, V.J., Crosatti, M., Nomura, A., Peek, R.M., Jr., Cover, T.L., Francois, F., and Blaser, M.J. (2007) Serological assays for identification of human gastric colonization by *Helicobacter pylori* strains expressing VacA m1 or m2. *Clinical and Vaccine Immunology* ***14***: 442-450.

Gomes, L.I., Rocha, G.A., Rocha, A.M., Soares, T.F., Oliveira, C.A., Bittencourt, P.F., and Queiroz, D.M. (2008) Lack of association between *Helicobacter pylori* infection with *dupA*-positive strains and gastroduodenal diseases in Brazilian patients. *International Journal of Medical Microbiology* ***298***: 223-230.

Goodwin, C.S., McCulloch, R.K., Armstrong, J.A., and Wee, S.H. (1985) Unusual cellular fatty acids and distinctive ultrastructure in a new spiral bacterium (*Campylobacter pyloridis*) from the human gastric mucosa. *Journal of Medical Microbiology* ***19***: 257-267.

Goodwin, C.S., Armstrong, J.A., Chilvers, T., Peters, M., Colins, M.D., Sly, L., McConnell, W., and Harper, W.E.S. (1989) Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov., respectively. *International Journal of Systematic Bacteriology* ***39***: 397-405.

Graham, D.Y., Opekun, A.R., Osato, M.S., El-Zimaity, H.M., Lee, C.K., Yamaoka, Y., Qureshi, W.A., Cadoz, M., and Monath, T.P. (2004) Challenge model for *Helicobacter pylori* infection in human volunteers. *Gut* **53**: 1235-1243.

Harder, K.W., Moller, N.P., Peacock, J.W., and Jirik, F.R. (1998) Protein-tyrosine phosphatase a regulates Src family kinases and alters cell-substratum adhesion. *Journal of Biological Chemistry* **273**: 31890-31900.

Harris, P.R., Cover, T.L., Crowe, D.R., Orenstein, J.M., Graham, M.F., Blaser, M.J., and Smith, P.D. (1996) *Helicobacter pylori* cytotoxin induces vacuolation of primary human mucosal epithelial cells. *Infection and Immunity* **64**: 4867-4871.

Hatakeyama, M. (2006) *Helicobacter pylori* CagA -- a bacterial intruder conspiring gastric carcinogenesis. *International Journal of Cancer* **119**: 1217-1223.

Hawtin, P.R., Stacey, A.R., and Newell, D.G. (1990) Investigation of the structure and localization of the urease of *Helicobacter pylori* using monoclonal antibodies. *Journal of General Microbiology* **136**: 1995-2000.

Hay, J.C. (2001) SNARE complex structure and function. *Experimental Cell Research* **271**: 10-21.

Hessey, S.J., Spencer, J., Wyatt, J.I., Sobala, G., Rathbone, B.J., Axon, A.T., and Dixon, M.F. (1990) Bacterial adhesion and disease activity in *Helicobacter*-associated chronic gastritis. *Gut* **31**: 134-138.

Higashi, H., Tsutsumi, R., Fujita, A., Yamazaki, S., Asaka, M., Azuma, T., and Hatakeyama, M. (2002a) Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. *Proceedings of the National Academy of Sciences (U.S.A.)* **99**: 14428-14433.

Higashi, H., Tsutsumi, R., Muto, S., Sugiyama, T., Azuma, T., Asaka, M., and Hatakeyama, M. (2002b) SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. *Science* **295**: 683-686.

Higashi, H., Nakaya, A., Tsutsumi, R., Yokoyama, K., Fujii, Y., Ishikawa, S., Higuchi, M., Takahashi, A., Kurashima, Y., Teishikata, Y., Tanaka, S., Azuma, T., and Hatakeyama, M. (2004) *Helicobacter pylori* CagA induces Ras-independent morphogenetic response through SHP-2 recruitment and activation. *Journal of Biological Chemistry* **279**: 17205-17216.

Higuchi, M., Tsutsumi, R., Higashi, H., and Hatakeyama, M. (2004) Conditional gene silencing utilizing the lac repressor reveals a role of SHP-2 in *cagA*-positive *Helicobacter pylori* pathogenicity. *Cancer Science* **95**: 442-447.

Hisatsune, J., Yamasaki, E., Nakayama, M., Shirasaka, D., Kurazono, H., Katagata, Y., Inoue, H., Han, J., Sap, J., Yahiro, K., Moss, J., and Hirayama, T. (2007) *Helicobacter pylori* VacA Enhances Prostaglandin E_2 Production through Induction of Cyclooxygenase 2 Expression via a p38 Mitogen-Activated Protein Kinase/Activating Transcription Factor 2 Cascade in AZ-521 Cells. *Infection and Immunity* **75**: 4472-4481.

Hoefen, R.J., and Berk, B.C. (2006) The multifunctional GIT family of proteins. *Journal of Cell Science* **119**: 1469-1475.

Holcombe, C. (1992) *Helicobacter pylori*: the African enigma. *Gut* **33**: 429-431.

Hotchin, N.A., Cover, T.L., and Akhtar, N. (2000) Cell vacuolation induced by the VacA cytotoxin of *Helicobacter pylori* is regulated by the Rac1 GTPase. *Journal of Biological Chemistry* **275**: 14009-14012.

Hussein, N.R., Mohammadi, M., Talebkhan, Y., Doraghi, M., Letley, D.P., Muhammad, M.K., Argent, R.H., and Atherton, J.C. (2008) Differences in virulence markers between *Helicobacter pylori* strains from Iraq and those from Iran: potential importance of regional differences in *H. pylori*-associated disease. *Journal of Clinical Microbiology* **46**: 1774-1779.

Hussein, N.R. (2010) The association of *dupA* and *Helicobacter pylori*-related gastroduodenal diseases. *European Journal of Clinical Microbiology and Infectious Diseases* **29**: 817821.

Ieva, R., and Bernstein, H.D. (2009) Interaction of an autotransporter passenger domain with BamA during its translocation across the bacterial outer membrane. *Proceedings of the National Academy of Sciences (U.S.A.)* **106**: 19120-19125.

Ikonomov, O.C., Sbrissa, D., Yoshimori, T., Cover, T.L., and Shisheva, A. (2002) PIKfyve Kinase and SKD1 AAA ATPase define distinct endocytic compartments. Only PIKfyve expression inhibits the cell-vacoulating activity of *Helicobacter pylori* VacA toxin. *Journal of Biological Chemistry* **277**: 46785-46790.

Ilver, D., Arnqvist, A., Ogren, J., Frick, I.M., Kersulyte, D., Incecik, E.T., Berg, D.E., Covacci, A., Engstrand, L., and Boren, T. (1998) *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science* **279**: 373-377.

Ilver, D., Barone, S., Mercati, D., Lupetti, P., and Telford, J.L. (2004) *Helicobacter pylori* toxin VacA is transferred to host cells via a novel contact-dependent mechanism. *Cellular Microbiology* **6**: 167-174.

Ismail, H., Balaratnam, N., Jenkins, G., Ismail, N., Williams, J., and Parry, J. (2001) The strong relationship between *Helicobacter pylori cagA* and *vacA* genotypes and disease outcome in a Saudi Arabian population. *Gut* **49**: A19.

Ismail, S., Hampton, M.B., and Keenan, J.I. (2003) *Helicobacter pylori* outer membrane vesicles modulate proliferation and interleukin-8 production by gastric epithelial cells. *Infection and Immunity* **71**: 5670-5675.

Ito, Y., Azuma, T., Ito, S., Miyaji, H., Hirai, M., Yamazaki, Y., Sato, F., Kato, T., Kohli, Y., and Kuriyama, M. (1997) Analysis and typing of the *vacA* gene from *cagA*-positive strains of *Helicobacter pylori* isolated in Japan. *Journal of Clinical Microbiology* **35**: 1710-1714.

Ivie, S.E., McClain, M.S., Algood, H.M., Lacy, D.B., and Cover, T.L. (2010) Analysis of a beta-helical region in the p55 domain of *Helicobacter pylori* vacuolating toxin. *BMC Microbiology* **10**: 60-69.

Iwamoto, H., Czajkowsky, D.M., Cover, T.L., Szabo, G., and Shao, Z. (1999) VacA from *Helicobacter pylori*: a hexameric chloride channel. *FEBS Letters* **450**: 101-104.

Jang, S., Jones, K.R., Olsen, C.H., Joo, Y.M., Yoo, Y.J., Chung, I.S., Cha, J.H., and Merrell, D.S. (2010) Epidemiological link between gastric disease and polymorphisms in VacA and CagA. *Journal of Clinical Microbiology* **48**: 559-567.

Jenks, P.J. (2006) *Helicobacter* spp. and related organisms. In *Principles and practice of clinical bacteriology, second edition*. Hawkey, P.M. (ed). Chichester: John Wiley & Sons, Ltd., pp. 473-483.

Ji, X., Fernandez, T., Burroni, D., Pagliaccia, C., Atherton, J.C., Reyrat, J.M., Rappuoli, R., and Telford, J.L. (2000) Cell specificity of the *Helicobacter pylori* cytotoxin is determined by a short region in the polymorphic midregion. *Infection and Immunity* **68**: 3754-3757.

Johnson, T.A., Qiu, J., Plaut, A.G., and Holyoak, T. (2009) Active-site gating regulates substrate selectivity in a chymotrypsin-like serine protease: the structure of *Haemophilus influenzae* immunoglobulin A1 protease. *Journal of Molecular Biology* **389**: 559-574.

Jones, K.R., Jang, S., Chang, J.Y., Kim, J., Chung, I.S., Olsen, C.H., Merrell, D.S., and Cha, J.H. (2011) Polymorphisms in the intermediate region of VacA impact *Helicobacter pylori*-induced disease development. *Journal of Clinical Microbiology* **49**: 101-110.

Jong, W.S., ten Hagen-Jongman, C.M., den Blaauwen, T., Slotboom, D.J., Tame, J.R., Wickstrom, D., de Gier, J.W., Otto, B.R., and Luirink, J. (2007) Limited tolerance towards folded elements during secretion of the autotransporter Hbp. *Molecular Microbiology* **63**: 1524-1536.

Jose, J., Wolk, U., Lorenzen, D., Wenschuh, H., and Meyer, T.F. (2000) Human T-cell response to meningococcal immunoglobulin A1 protease associated alpha-proteins. *Scandinavian Journal of Immunology* **51**: 176-185.

Junker, M., Schuster, C.C., McDonnell, A.V., Sorg, K.A., Finn, M.C., Berger, B., and Clark, P.L. (2006) Pertactin beta-helix folding mechanism suggests common themes for the secretion and folding of autotransporter proteins. *Proceedings of the National Academy of Sciences (U.S.A.)* **103**: 4918-4923.

Junker, M., Besingi, R.N., and Clark, P.L. (2009) Vectorial transport and folding of an autotransporter virulence protein during outer membrane secretion. *Molecular Microbiology* **71**: 1323-1332.

Kabir, S. (2007) The current status of *Helicobacter pylori* vaccines: a review. *Helicobacter* **12**: 89-102.

Kawachi, H., Fujikawa, A., Maeda, N., and Noda, M. (2001) Identification of GIT1/Cat-1 as a substrate molecule of protein tyrosine phosphatase □ /□ by the yeast substrate-trapping

system. *Proceedings of the National Academy of Sciences (U.S.A.)* **98**: 6593-6598.

Keenan, J., Day, T., Neal, S., Cook, B., Perez-Perez, G., Allardyce, R., and Bagshaw, P. (2000) A role for the bacterial outer membrane in the pathogenesis of *Helicobacter pylori* infection. *FEMS Microbiology Letters* **182**: 259-264.

Kidd, M., Lastovica, A.J., Atherton, J.C., and Louw, J.A. (1999) Heterogeneity in the *Helicobacter pylori vacA* and *cagA* genes: association with gastroduodenal disease in South Africa? *Gut* **45**: 499-502.

Kim, S., Chamberlain, A.K., and Bowie, J.U. (2004) Membrane channel structure of *Helicobacter pylori* vacuolating toxin: role of multiple GXXXG motifs in cylindrical channels. *Proceedings of the National Academy of Sciences (U.S.A.)* **101**: 5988-5991.

Kimura, M., Goto, S., Wada, A., Yahiro, K., Niidome, T., Hatakeyama, T., Aoyagi, H., Hirayama, T., and Kondo, T. (1999) Vacuolating cytotoxin purified from *Helicobacter pylori* causes mitochondrial damage in human gastric cells. *Microbial Pathogenesis* **26**: 45-52.

Kirchhausen, T. (1998) Vesicle formation: dynamic dynamin lives up to its name. *Current Biology* **8**: R792-R794.

Kobayashi, K., Sakamoto, J., Kito, T., Yamamura, Y., Koshikawa, T., Fujita, M., Watanabe, T., and Nakazato, H. (1993) Lewis blood group-related antigen expression in normal gastric epithelium, intestinal metaplasia, gastric adenoma, and gastric carcinoma. *American Journal of Gastroenterology* **88**: 919-924.

Kodama, K., Ito, A., Nishizono, A., Fujioka, T., Nasu, M., Yahiro, K., Hirayama, T., and Uemura, N. (1999) Divergence of virulence factors of *Helicobacter pylori* among clinical isolates does not correlate with disease specificity. *Journal of Gastroenterology* **34 (Suppl 11)**: 6-9.

Krienitz, W. (1906) Ueber das Auftreten von Spirochäten verschiedener Form im Mageninhalt bei Carcinoma ventriculi. *Dtsch Med Wochenschr* **28**: 872.

Kuck, D., Kolmerer, B., Iking-Konert, C., Krammer, P.H., Stremmel, W., and Rudi, J. (2001) Vacuolating cytotoxin of *Helicobacter pylori* induces apoptosis in the human gastric epithelial cell line AGS. *Infection and Immunity* **69**: 5080-5087.

Kudo, T., Lu, H., Wu, J.Y., Graham, D.Y., Casola, A., and Yamaoka, Y. (2005) Regulation of RANTES promoter activation in gastric epithelial cells infected with *Helicobacter pylori*. *Infection and Immunity* **73**: 7602-7612.

Kuipers, E.J., Thijs, J.C., and Festen, H.P. (1995) The prevalence of *Helicobacter pylori* in peptic ulcer disease. *Alimentary Pharmacology and Therapeutics* **9 (Suppl 2)**: 59-69.

Kuo, C.H., and Wang, W.C. (2003) Binding and internalization of *Helicobacter pylori* VacA via cellular lipid rafts in epithelial cells. *Biochemical and Biophysical Research Communications* **303**: 640-644.

Kusters, J.G., van Vliet, A.H., and Kuipers, E.J. (2006) Pathogenesis of *Helicobacter pylori* infection. *Clinical Microbiology Reviews* **19**: 449-490.

Lanzavecchia, S., Bellon, P.L., Lupetti, P., Dallai, R., Rappuoli, R., and Telford, J.L. (1998) Three-dimensional reconstruction of metal replicas of the *Helicobacter pylori* vacuolating cytotoxin. *Journal of Structural Biology* **121**: 9-18.

Letley, D.P., Lastovica, A., Louw, J.A., Hawkey, C.J., and Atherton, J.C. (1999) Allelic diversity of the *Helicobacter pylori* vacuolating cytotoxin gene in South Africa: rarity of

the *vacA* s1a genotype and natural occurrence of an s2/m1 allele. *Journal of Clinical Microbiology* **37**: 1203-1205.

Letley, D.P., and Atherton, J.C. (2000) Natural diversity in the N terminus of the mature vacuolating cytotoxin of *Helicobacter pylori* determines cytotoxin activity. *Journal of Bacteriology* **182**: 3278-3280.

Letley, D.P., Rhead, J.L., Twells, R.J., Dove, B., and Atherton, J.C. (2003) Determinants of nontoxicity in the gastric pathogen *Helicobacter pylori*. *Journal of Biological Chemistry* **278**: 26734-26741.

Letley, D.P., Rhead, J.L., Bishop, K., and Atherton, J.C. (2006) Paired cysteine residues are required for high levels of the *Helicobacter pylori* autotransporter VacA. *Microbiology* **152**: 1319-1325.

Leung, W.K., Lin, S.R., Ching, J.Y., To, K.F., Ng, E.K., Chan, F.K., Lau, J.Y., and Sung, J.J. (2004) Factors predicting progression of gastric intestinal metaplasia: results of a randomised trial on *Helicobacter pylori* eradication. *Gut* **53**: 1244-1249.

Leunk, R.D., Johnson, P.T., David, B.C., Kraft, W.G., and Morgan, D.R. (1988) Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *Journal of Medical Microbiology* **26**: 93-99.

Linden, S., Nordman, H., Hedenbro, J., Hurtig, M., Boren, T., and Carlstedt, I. (2002) Strain-and blood group-dependent binding of *Helicobacter pylori* to human gastric MUC5AC glycoforms. *Gastroenterology* **123**: 1923-1930.

Loveless, B.J., and Saier, M.H. (1997) A novel family of channel-forming, autotransporting, bacterial virulence factors. *Molecular Membrane Biology* **14**: 113-123.

Lu, H., Hsu, P.I., Graham, D.Y., and Yamaoka, Y. (2005a) Duodenal ulcer promoting gene of *Helicobacter pylori*. *Gastroenterology* **128**: 833-848.

Lu, H., Wu, J.Y., Kudo, T., Ohno, T., Graham, D.Y., and Yamaoka, Y. (2005b) Regulation of interleukin-6 promoter activation in gastric epithelial cells infected with *Helicobacter pylori*. *Molecular Biology of the Cell* **16**: 4954-4966.

Lu, H., Wu, J.Y., Beswick, E.J., Ohno, T., Odenbreit, S., Haas, R., Reyes, V.E., Kita, M., Graham, D.Y., and Yamaoka, Y. (2007) Functional and intracellular signaling differences associated with the *Helicobacter pylori* AlpAB adhesin from Western and East Asian strains. *Journal of Biological Chemistry* **282**: 6242-6254.

Lupetti, P., Heuser, J.E., Manetti, R., Massari, P., Lanzavecchia, S., Bellon, P.L., Dallai, R., Rappuoli, R., and Telford, J.L. (1996) Oligomeric and subunit structure of the *Helicobacter pylori* vacuolating cytotoxin. *Journal Of Cell Biology* **133**: 801-807.

Mahdavi, J., Sonden, B., Hurtig, M., Olfat, F.O., Forsberg, L., Roche, N., Angstrom, J., Larsson, T., Teneberg, S., Karlsson, K.A., Altraja, S., Wadstrom, T., Kersulyte, D., Berg, D.E., Dubois, A., Petersson, C., Magnusson, K.E., Norberg, T., Lindh, F., Lundskog, B.B., Arnqvist, A., Hammarstrom, L., and Boren, T. (2002) *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. *Science* **297**: 573-578.

Malaty, H.M. (2007) Epidemiology of *Helicobacter pylori* infection. *Best Practice and Research Clinical Gastroenterology* **21**: 205-214.

Manetti, R., Massari, P., Burroni, D., de Bernard, M., Marchini, A., Olivieri, R., Papini, E., Montecucco, C., Rappuoli, R., and Telford, J.L. (1995) *Helicobacter pylori* cytotoxin: Importance of native conformation for induction of neutralizing antibodies. *Infection and Immunity* **63**: 4476-4480.

Marcos, N.T., Magalhaes, A., Ferreira, B., Oliveira, M.J., Carvalho, A.S., Mendes, N., Gilmartin, T., Head, S.R., Figueiredo, C., David, L., Santos-Silva, F., and Reis, C.A. (2008) *Helicobacter pylori* induces beta3GnT5 in human gastric cell lines, modulating expression of the SabA ligand sialyl-Lewis x. *Journal of Clinical Investigation* **118**: 2325-2336.

Marcus, E.A., Moshfegh, A.P., Sachs, G., and Scott, D.R. (2005) The periplasmic a-carbonic anhydrase activity of *Helicobacter pylori* is essential for acid acclimation. *Journal of Bacteriology* **187**: 729-738.

Marshall, B.J., and Warren, J.R. (1984) Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **1**: 1311-1315.

Marshall, B.J., Armstrong, J.A., McGechie, D.B., and Glancy, R.J. (1985) Attempt to fulfil Koch's postulates for pyloric *Campylobacter*. *Medical Journal of Australia* **142**: 436-439.

Marshall, B.J., Barrett, L.J., Prakash, C., McCallum, R.W., and Guerrant, R.L. (1990) Urea protects *Helicobacter* (*Campylobacter*) *pylori* from the bactericidal effect of acid. *Gastroenterology* **99**: 697-702.

Martinez, T.A., Gonzalez, C.C., Kawaguchi, P.F., Montoya, R., Corvalan, A., Madariaga, J., Roa, J., Garcia, C.A., Salgado, F., Solar, H., and Palma, M. (2001) *Helicobacter pylor*i: *cagA* status and *vacA* genotyping in Chile. Detection of a s2/m1 strain. *Revista Medica De Chile* **129**: 1147-1153.

Massari, P., Manetti, R., Burroni, D., Nuti, S., Norais, N., Rappuoli, R., and Telford, J.L. (1998) Binding of the *Helicobacter pylori* vacuolating cytotoxin to target cells. *Infection and Immunity* **66**: 3981-3984.

May, K.L., and Morona, R. (2008) Mutagenesis of the *Shigella flexneri* autotransporter IcsA reveals novel functional regions involved in IcsA biogenesis and recruitment of host neural Wiscott-Aldrich syndrome protein. *Journal of Bacteriology* **190**: 4666-4676.

McClain, M.S., Schraw, W., Ricci, V., Boquet, P., and Cover, T.L. (2000) Acid activation of *Helicobacter pylori* vacuolating cytotoxin (VacA) results in toxin internalization by eukaryotic cells. *Molecular Microbiology* **37**: 433-442.

McClain, M.S., Cao, P., and Cover, T.L. (2001a) Amino-terminal hydrophobic region of *Helicobacter pylori* vacuolating cytotoxin (VacA) mediates transmembrane protein dimerization. *Infection and Immunity* **69**: 1181-1184.

McClain, M.S., Cao, P., Iwamoto, H., Vinion-Dubiel, A.D., Szabo, G., Shao, Z., and Cover, T.L. (2001b) The 12-amino-acid segment, present in type s2 but not type s1 *Helicobacter*

pylori VacA proteins, abolishes cytotoxin activity and alters membrane channel formation. *Journal of Bacteriology* **183**: 6499-6508.

McClain, M.S., Iwamoto, H., Cao, P., Vinion-Dubiel, A.D., Li, Y., Szabo, G., Shao, Z., and Cover, T.L. (2003) Essential role of a G*XXX*G motif for membrane channel formation by *Helicobacter pylori* vacuolating toxin. *Journal of Biological Chemistry* **278**: 12101-12108.

McClain, M.S., Czajkowsky, D.M., Torres, V.J., Szabo, G., Shao, Z., and Cover, T.L. (2006) Random mutagenesis of *Helicobacter pylori vacA* to identify amino acids essential for vacuolating cytotoxic activity. *Infection and Immunity* **74**: 6188-6195.

McGee, D.J., Radcliff, F.J., Mendz, G.L., Ferrero, R.L., and Mobley, H.L. (1999) *Helicobacter pylori rocF* is required for arginase activity and acid protection *in vitro* but is not essential for colonization of mice or for urease activity. *Journal of Bacteriology* **181**: 7314-7322.

McGee, D.J., Langford, M.L., Watson, E.L., Carter, J.E., Chen, Y.T., and Ottemann, K.M. (2005) Colonization and inflammation deficiencies in Mongolian gerbils infected by *Helicobacter pylori* chemotaxis mutants. *Infection and Immunity* **73**: 1820-1827.

McNamara, D., and O'Morain, C. (1999) Gastro-oesophageal reflux disease and *Helicobacter pylori*: an intricate relation. *Gut* **45 Suppl 1**: I13-I17.

Meng, K., Rodriguez-Peña, A., Dimitrov, T., Chen, W., Yamin, M., Noda, M., and Deuel, T.F. (2000) Pleiotrophin signals increased tyrosine phosphorylation of 13-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase 3/□. *Proceedings of the National Academy of Sciences (U.S.A.)* **97**: 26032608.

Mera, R., Fontham, E.T., Bravo, L.E., Bravo, J.C., Piazuelo, M.B., Camargo, M.C., and Correa, P. (2005) Long term follow up of patients treated for *Helicobacter pylori* infection. *Gut* **54**: 1536-1540.

Meyer, F., Ramanujam, K.S., Gobert, A.P., James, S.P., and Wilson, K.T. (2003) Cutting edge: cyclooxygenase-2 activation suppresses Th1 polarization in response to *Helicobacter pylori*. *Journal of Immunology* **171**: 3913-3917.

Miehlke, S., Kirsch, C., Agha-Amiri, K., Günther, T., Lehn, N., Malfertheiner, P., Stolte, M., Ehninger, G., and Bayerdörffer, E. (2000) The *Helicobacter pylori vacA* s1, m1 genotype and *cagA* is associated with gastric carcinoma in Germany. *International Journal of Cancer* **87**: 322-327.

Mimuro, H., Suzuki, T., Tanaka, J., Asahi, M., Haas, R., and Sasakawa, C. (2002) Grb2 is a key mediator of *Helicobacter pylori* CagA protein activities. *Molecular Cell* **10**: 745-755.

Mizote, T., Yoshiyama, H., and Nakazawa, T. (1997) Urease-independent chemotactic responses of *Helicobacter pylori* to urea, urease inhibitors, and sodium bicarbonate. *Infection and Immunity* **65**: 1519-1521.

Molinari, M., Galli, C., Norais, N., Telford, J.L., Rappuoli, R., Luzio, J.P., and Montecucco, C. (1997) Vacuoles induced by *Helicobacter pylori* toxin contain both late endosomal and lysosomal markers. *Journal of Biological Chemistry* **272**: 25339-25344.

Molinari, M., Galli, C., de Bernard, M., Norais, N., Ruysschaert, J.-M., Rappuoli, R., and Montecucco, C. (1998a) The acid activation of *Helicobacter pylori* toxin VacA: structural and membrane binding studies. *Biochemical and Biophysical Research Communications* **248**: 334-340.

Molinari, M., Salio, M., Galli, C., Norais, N., Rappuoli, R., Lanzavecchia, A., and Montecucco, C. (1998b) Selective inhibition of Ii-dependent antigen presentation by *Helicobacter pylori* toxin VacA. *Journal of Experimental Medicine* **187**: 135-140.

Moll, G., Papini, E., Colonna, R., Burroni, D., Telford, J., Rappuoli, R., and Montecucco, C. (1995) Lipid interaction of the 37 kDa and 58 kDa fragments of the *Helicobacter pylori* cytotoxin. *European Journal of Biochemistry* **234**: 947-952.

Morales-Espinosa, R., Castillo-Rojas, G., Gonzalez-Valencia, G., de Leon, S.P., Cravioto, A., Atherton, J.C., and Lopez-Vidal, Y. (1999) Colonization of Mexican patients by multiple *Helicobacter pylori* strains with different *vacA* and *cagA* genotypes. *Journal of Clinical Microbiology* **37**: 3001-3004.

Moran, A.P., Lindner, B., and Walsh, E.J. (1997) Structural characterization of the lipid A component of *Helicobacter pylori* rough- and smooth-form lipopolysaccharides. *Journal of Bacteriology* **179**: 6453-6463.

Moran, A.P. (2007) Lipopolysaccharide in bacterial chronic infection: insights from *Helicobacter pylori* lipopolysaccharide and lipid A. *International Journal of Medical Microbiology* **297**: 307-319.

Morgan, D.R., Freedman, R., Depew, C.E., and Kraft, W.G. (1987) Growth of *Campylobacter pylori* in liquid media. *Journal of Clinical Microbiology* **25**: 2123-2125.

Morgner, A., Schmelz, R., Thiede, C., Stolte, M., and Miehlke, S. (2007) Therapy of gastric mucosa associated lymphoid tissue lymphoma. *World Journal of Gastroenterology* **13**: 3554-3566.

Morris, A., and Nicholson, G. (1987) Ingestion of *Campylobacter pyloridis* causes gastritis and raised fasting gastric pH. *American Journal of Gastroenterology* **82**: 192-199.

Morris, A.J., Ali, M.R., Nicholson, G.I., Perez-Perez, G.I., and Blaser, M.J. (1991) Long-term follow-up of voluntary ingestion of *Helicobacter pylori*. *Annals of Internal Medicine* **114**: 662-663.

Mullock, B.M., Smith, C.W., Ihrke, G., Bright, N.A., Lindsay, M., Parkinson, E.J., Brooks, D.A., Parton, R.G., James, D.E., Luzio, J.P., and Piper, R.C. (2000) Syntaxin 7 is localized to late endosome compartments, associates with Vamp 8, and is required for late endosome-lysosome fusion. *Molecular Biology of the Cell* **11**: 3137-3153.

Muotiala, A., Helander, I.M., Pyhala, L., Kosunen, T.U., and Moran, A.P. (1992) Low biological activity of *Helicobacter pylori* lipopolysaccharide. *Infection and Immunity* **60**: 1714-1716.

Nakamura, N., Yamamoto, A., Wada, Y., and Futai, M. (2000) Syntaxin 7 mediates endocytic trafficking to late endosomes. *J Biol Chem* **275**: 6523-6529.

Nakayama, M., Kimura, M., Wada, A., Yahiro, K., Ogushi, K., Niidome, T., Fujikawa, A., Shirasaka, D., Aoyama, N., Kurazono, H., Noda, M., Moss, J., and Hirayama, T. (2004) *Helicobacter pylori* VacA activates the p38/activating transcription factor 2-mediated signal pathway in AZ-521 cells. *Journal of Biological Chemistry* **279**: 7024-7028.

Nakayama, M., Hisatsune, J., Yamasaki, E., Nishi, Y., Wada, A., Kurazono, H., Sap, J., Yahiro, K., Moss, J., and Hirayama, T. (2006) Clustering of *Helicobacter pylori* VacA in lipid rafts, mediated by its receptor, receptor-like protein tyrosine phosphatase 0, is required for intoxication in AZ-521 Cells. *Infection and Immunity* **74**: 6571-6580.

Nguyen, V.Q., Caprioli, R.M., and Cover, T.L. (2001) Carboxy-terminal proteolytic processing of *Helicobacter pylori* vacuolating toxin. *Infection and Immunity* **69**: 543-546.

Odenbreit, S., Puls, J., Sedlmaier, B., Gerland, E., Fischer, W., and Haas, R. (2000) Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science* **287**: 1497-1500.

Odenbreit, S., Faller, G., and Haas, R. (2002a) Role of the *alpAB* proteins and lipopolysaccharide in adhesion of *Helicobacter pylori* to human gastric tissue. *International Journal of Medical Microbiology* **292**: 247-256.

Odenbreit, S., Kavermann, H., Puls, J., and Haas, R. (2002b) CagA tyrosine phosphorylation and interleukin-8 induction by *Helicobacter pylori* are independent from *alpAB*, HopZ and *bab* group outer membrane proteins. *International Journal of Medical Microbiology* **292**: 257-266.

Ogiwara, H., Sugimoto, M., Ohno, T., Vilaichone, R.K., Mahachai, V., Graham, D.Y., and Yamaoka, Y. (2009) Role of deletion located between the intermediate and middle regions of the *Helicobacter pylori vacA* gene in cases of gastroduodenal diseases. *Journal of Clinical Microbiology* **47**: 3493-3500.

Oldani, A., Cormont, M., Hofman, V., Chiozzi, V., Oregioni, O., Canonici, A., Sciullo, A., Sommi, P., Fabbri, A., Ricci, V., and Boquet, P. (2009) *Helicobacter pylori* counteracts the apoptotic action of its VacA toxin by injecting the CagA protein into gastric epithelial cells. *PLoS Pathogens* **5**: e1000603.

Oliver, D.C., Huang, G., Nodel, E., Pleasance, S., and Fernandez, R.C. (2003) A conserved region within the *Bordetella pertussis* autotransporter BrkA is necessary for folding of its passenger domain. *Molecular Microbiology* **47**: 1367-1383.

Padilla, P.I., Wada, A., Yahiro, K., Kimura, M., Niidome, T., Aoyagi, H., Kumatori, A., Anami, M., Hayashi, T., Fujisawa, J., Saito, H., Moss, J., and Hirayama, T. (2000) Morphologic differentiation of HL-60 cells is associated with appearance of RPTP|3 and induction of *Helicobacter pylori* VacA sensitivity. *Journal of Biological Chemistry* **275**: 15200-15206.

Pagliaccia, C., de Bernard, M., Lupetti, P., Ji, X., Burroni, D., Cover, T.L., Papini, E., Rappuoli, R., Telford, J.L., and Reyrat, J.M. (1998) The m2 form of the *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. *Proceedings of the National Academy of Sciences (U.S.A.)* **95**: 10212-10217.

Panayotopoulou, E.G., Sgouras, D.N., Papadakos, K.S., Petraki, K., Breurec, S., Michopoulos, S., Mantzaris, G., Papatheodoridis, G., Mentis, A., and Archimandritis, A. (2010) CagA and VacA polymorphisms are associated with distinct pathological features in *Helicobacter pylori*-infected adults with peptic ulcer and non-peptic ulcer disease. *Journal of Clinical Microbiology* **48**: 2237-2239.

Papini, E., Bugnoli, M., de Bernard, M., Figura, N., Rappuoli, R., and Montecucco, C. (1993a) Bafilomycin A1 inhibits *Helicobacter pylori*-induced vacuolization of HeLa cells. *Molecular Microbiology* **7**: 323-327.

Papini, E., de Bernard, M., Bugnoli, M., Milia, E., Rappuoli, R., and Montecucco, C. (1993b) Cell vacuolation induced by *Helicobacter pylori*: inhibition by bafilomycins A1, B1, C1 and D. *FEMS Microbiology Letters* **113**: 155-160.

Papini, E., de Bernard, M., Milia, E., Bugnoli, M., Zerial, M., Rappuoli, R., and Montecucco, C. (1994) Cellular vacuoles induced by *Helicobacter pylori* originate from late endosomal compartments. *Proceedings of the National Academy of Sciences (U.S.A.)* **91**: 97209724.

Papini, E., Gottardi, E., Satin, B., de Bernard, M., Massari, P., Telford, J., Rappuoli, R., Sato, S.B., and Montecucco, C. (1996) The vacuolar ATPase proton pump is present on intracellular vacuoles induced by *Helicobacter pylori*. *Journal of Medical Microbiology* **45**: 84-89.

Papini, E., Satin, B., Bucci, C., de Bernard, M., Telford, J.L., Manetti, R., Rappuoli, R., Zerial, M., and Montecucco, C. (1997) The small GTP binding protein rab7 is essential for cellular vacuolation induced by *Helicobacter pylori* cytotoxin. *EMBO Journal* **16**: 15-24.

Papini, E., Satin, B., Norais, N., de Bernard, M., Telford, J.L., Rappuoli, R., and Montecucco, C. (1998) Selective increase of the permeability of polarized epithelial cell monolayers by *Helicobacter pylori* vacuolating toxin. *Journal of Clinical Investigation* **102**: 813-820.

Parsonnet, J., Friedman, G.D., Vandersteen, D.P., Chang, Y., Vogelman, J.H., Orentreich, N., and Sibley, R.K. (1991a) *Helicobacter pylori* infection and the risk of gastric carcinoma. *New England Journal of Medicine* **325**: 1127-1131.

Parsonnet, J., Vandersteen, D., Goates, J., Sibley, R.K., Pritikin, J., and Chang, Y. (1991b) *Helicobacter pylori* infection in intestinal- and diffuse-type gastric adenocarcinomas. *Journal of the National Cancer Institute* **83**: 640-643.

Parsonnet, J., Hansen, S., Rodriguez, L., Gelb, A., Warnke, R., and Jellum, E. (1994) *Helicobacter* infection and gastric lymphoma. *New England Journal of Medicine* **330**: 1267-1271.

Peek, R.M., Jr., and Crabtree, J.E. (2006) *Helicobacter* infection and gastric neoplasia. *Journal of Pathology* **208**: 233-248.

Pelicic, V., Reyrat, J.M., Sartori, L., Pagliaccia, C., Rappuoli, R., Telford, J.L., Montecucco, C., and Papini, E. (1999) *Helicobacter pylori* VacA cytotoxin associated with the bacteria increases epithelial permeability independently of its vacuolating activity. *Microbiology* **145**: 2043-2050.

Pellicano, A., Imeneo, M., Leone, I., Larussa, T., and Luzza, F. (2007) Enhanced activation of cyclooxygenase-2 downregulates Th1 signaling pathway in *Helicobacter pylori*-infected human gastric mucosa. *Helicobacter* **12**: 193-199.

Pereira Lage, A., Glupczynski, Y., Goossens, H., Burette, A., and Butzler, J.-P. (1993) Neutralising antibody response to the vacuolating toxin of *Helicobacter pylori* in gastritis only and peptic ulcer patients. *Zentralblatt für Bakteriologie : international journal of medical microbiology* **280**: 197-202.

Pérez-Pérez, G.I., Peek, R.M., Jr., Atherton, J.C., Blaser, M.J., and Cover, T.L. (1999) Detection of anti-VacA antibody responses in serum and gastric juice samples using type s1/m1 and s2/m2 *Helicobacter pylori* VacA antigens. *Clinical and Diagnostic Laboratory Immunology* **6**: 489-493.

Petersson, C., Forsberg, M., Aspholm, M., Olfat, F.O., Forslund, T., Boren, T., and Magnusson, K.E. (2006) *Helicobacter pylori* SabA adhesin evokes a strong inflammatory response in human neutrophils which is down-regulated by the neutrophil-activating protein. *Medical Microbiology and Immunology* **195**: 195-206.

Pflock, M., Kennard, S., Delany, I., Scarlato, V., and Beier, D. (2005) Acid-induced activation of the urease promoters is mediated directly by the ArsRS two-component system of *Helicobacter pylori*. *Infection and Immunity* **73**: 6437-6445.

Pflock, M., Finsterer, N., Joseph, B., Mollenkopf, H., Meyer, T.F., and Beier, D. (2006) Characterization of the ArsRS regulon of *Helicobacter pylori*, involved in acid adaptation. *Journal of Bacteriology* **188**: 3449-3462.

Phadnis, S.H., Ilver, D., Janzon, L., Normark, S., and Westblom, T.U. (1994) Pathological significance and molecular characterisation of the vacuolating cytotoxin gene of *Helicobacter pylori*. *Infection and Immunity* **62**: 1557-1565.

Pohlner, J., Halter, R., Beyreuther, K., and Meyer, T.F. (1987) Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. *Nature* **325**: 458-462.

Pohlner, J., Langenberg, U., Wolk, U., Beck, S.C., and Meyer, T.F. (1995) Uptake and nuclear transport of *Neisseria* IgA1 protease-associated alpha-proteins in human cells. *Molecular Microbiology* **17**: 1073-1083.

Prinz, C., Schoniger, M., Rad, R., Becker, I., Keiditsch, E., Wagenpfeil, S., Classen, M., Rosch, T., Schepp, W., and Gerhard, M. (2001) Key importance of the *Helicobacter pylori* adherence factor blood group antigen binding adhesin during chronic gastric inflammation. *Cancer Research* **61**: 1903-1909.

Qiao, W., Hu, J.L., Xiao, B., Wu, K.C., Peng, D.R., Atherton, J.C., and Xue, H. (2003) *cagA* and *vacA* genotype of *Helicobacter pylori* associated with gastric diseases in Xi'an area. *World Journal of Gastroenterology* **9**: 1762-1766.

Rad, R., Gerhard, M., Lang, R., Schoniger, M., Rosch, T., Schepp, W., Becker, I., Wagner, H., and Prinz, C. (2002) The *Helicobacter pylori* blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. *Journal of Immunology* **168**: 3033-3041.

Radin, J.N., Gonzalez-Rivera, C., Ivie, S.E., McClain, M.S., and Cover, T.L. (2011) *Helicobacter pylori* VacA induces programmed necrosis in gastric epithelial cells. *Infection and Immunity*: doi:10.1128/IAI.01370-01310.

Raeiszadeh, M., Fernando, N., Holton, J., Vaira, D., Siavoshi, F., Hosseini, A., and Kelly, P. (2003) Variation in *Helicobacter pylori* strains from Iran and Zambia. *Gut* ***52***: 289.

Rajasekaran, A.K., Hojo, M., Huima, T., and Rodriguez-Boulan, E. (1996) Catenins and zonula occludens-1 form a complex during early stages in the assembly of tight junctions. *Journal of Cell Biology* ***132***: 451-463.

Rautelin, H., Blomberg, B., Jarnerot, G., and Danielsson, D. (1994) Nonopsonic activation of neutrophils and cytotoxin production by *Helicobacter pylori*: ulcerogenic markers. *Scandinavian Journal of Gastroenterology* ***29***: 128-132.

Renn, J.P., and Clark, P.L. (2008) A conserved stable core structure in the passenger domain beta-helix of autotransporter virulence proteins. *Biopolymers* ***89***: 420-427.

Reyrat, J.M., Lanzavecchia, S., Lupetti, P., de Bernard, M., Pagliaccia, C., Pelicic, V., Charrel, M., Ulivieri, C., Norais, N., Ji, X., Cabiaux, V., Papini, E., Rappuoli, R., and Telford, J.L. (1999) 3D imaging of the 58 kDa cell binding subunit of the *Helicobacter pylori* cytotoxin. *Journal of Molecular Biology* ***290***: 459-470.

Rhead, J.L., Letley, D.P., Mohammadi, M., Hussein, N., Mohagheghi, M.A., Eshagh Hosseini, M., and Atherton, J.C. (2007) A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. *Gastroenterology* ***133***: 926-936.

Ricci, V., Galmiche, A., Doye, A., Necchi, V., Solcia, E., and Boquet, P. (2000) High cell sensitivity to *Helicobacter pylori* VacA toxin depends on a GPI-anchored protein and is not blocked by inhibition of the clathrin-mediated pathway of endocytosis. *Molecular Biology of the Cell* ***11***: 3897-3909.

Ricci, V., Chiozzi, V., Necchi, V., Oldani, A., Romano, M., Solcia, E., and Ventura, U. (2005) Free-soluble and outer membrane vesicle-associated VacA from *Helicobacter pylori*: two forms of release, a different activity. *Biochemical and Biophysical Research Communications* ***337***: 173-178.

Roche, N., Ilver, D., Ångström, J., Barone, S., Telford, J.L., and Teneberg, S. (2007) Human gastric glycosphingolipids recognized by *Helicobacter pylori* vacuolating cytotoxin VacA. *Microbes and Infection* ***9***: 605-614.

Rudi, J., Kolb, C., Maiwald, M., Kuck, D., Sieg, A., Galle, P.R., and Stremmel, W. (1998) Diversity of *Helicobacter pylori vacA* and *cagA* genes and relationship to VacA and CagA protein expression, cytotoxin production, and associated diseases. *Journal of Clinical Microbiology* ***36***: 944-948.

Russ, W.P., and Engelman, D.M. (2000) The GxxxG motif: a framework for transmembrane helix-helix association. *Journal of Molecular Biology* ***296***: 911-919.

Sabharanjak, S., Sharma, P., Parton, R.G., and Mayor, S. (2002) GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Developmental Cell* **2**: 411-423.

Sadakane, Y., Kusaba, K., Nagasawa, Z., Tanabe, I., Kuroki, S., and Tadano, J. (1999) Prevalence and genetic diversity of *cagD*, *cagE*, and *vacA* in *Helicobacter pylori* strains isolated from Japanese patients. *Scandinavian Journal of Gastroenterology* **34**: 981986.

Sakamoto, S., Watanabe, T., Tokumaru, T., Takagi, H., Nakazato, H., and Lloyd, K.O. (1989) Expression of Lewisa, Lewisb, Lewisx, Lewisy, siayl-Lewisa, and sialyl-Lewisx blood group antigens in human gastric carcinoma and in normal gastric tissue. *Cancer Research* **49**: 745-752.

Salama, N.R., Otto, G., Tompkins, L., and Falkow, S. (2001) Vacuolating cytotoxin of *Helicobacter pylori* plays a role during colonization in a mouse model of infection. *Infection and Immunity* **69**: 730-736.

Sap, J., D'Eustachio, P., Givol, D., and Schlessinger, J. (1990) Cloning and expression of a widely expressed receptor tyrosine phosphatase. *Proceedings of the National Academy of Sciences (U.S.A.)* **87**: 6112-6116.

Satin, B., Norais, N., Telford, J., Rappuoli, R., Murgia, M., Montecucco, C., and Papini, E. (1997) Effect of *Helicobacter pylori* vacuolating toxin on maturation and extracellular release of procathepsin D and on epidermal growth factor degradation. *Journal of Biological Chemistry* **272**: 25022-25028.

Schmees, C., Prinz, C., Treptau, T., Rad, R., Hengst, L., Voland, P., Bauer, S., Brenner, L., Schmid, R.M., and Gerhard, M. (2007) Inhibition of T-cell proliferation by *Helicobacter pylori* γ-glutamyl transpeptidase. *Gastroenterology* **132**: 1820-1833.

Schmitt, W., and Haas, R. (1994) Genetic analysis of the *Helicobacter pylori* vacuolating cytotoxin: structural similarities with the IgA protease type of exported protein. *Molecular Microbiology* **12**: 307-319.

Schraw, W., Li, Y., McClain, M.S., van der Goot, F.G., and Cover, T.L. (2002) Association of *Helicobacter pylori* vacuolating toxin (VacA) with lipid rafts. *Journal of Biological Chemistry* **277**: 34642-34650.

Scott, D.R., Weeks, D., Hong, C., Postius, S., Melchers, K., and Sachs, G. (1998) The role of internal urease in acid resistance of *Helicobacter pylori*. *Gastroenterology* **114**: 58-70.

Segal, E.D., Cha, J., Lo, J., Falkow, S., and Tompkins, L.S. (1999) Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *Proceedings of the National Academy of Sciences (U.S.A.)* **96**: 14559-14564.

Selbach, M., Moese, S., Hauck, C.R., Meyer, T.F., and Backert, S. (2002) Src is the kinase of the *Helicobacter pylori* CagA protein *in vitro* and *in vivo*. *Journal of Biological Chemistry* **277**: 6775-6778.

Selbach, M., Moese, S., Hurwitz, R., Hauck, C.R., Meyer, T.F., and Backert, S. (2003) The *Helicobacter pylori* CagA protein induces cortactin dephosphorylation and actin rearrangement by c-Src inactivation. *EMBO Journal* **22**: 515-528.

Shang, J., and Peña, A.S. (2005) Multidisciplinary approach to understand the pathogenesis of gastric cancer. *World Journal of Gastroenterology* **11**: 4131-4139.

Sheu, S.M., Hung, K.H., Sheu, B.S., Yang, H.B., and Wu, J.J. (2009) Association of nonsynonymous substitutions in the intermediate region of the *vacA* gene of *Helicobacter pylori* with gastric diseases in Taiwan. *Journal of Clinical Microbiology* **47**: 249-251.

Shimoyama, T., Yoshimura, T., Mikami, T., Fukuda, S., Crabtree, J.E., and Munakata, A. (1998) Evaluation of *Helicobacter pylori vacA* genotype in Japanese patients with gastric cancer. *Journal of Clinical Pathology* **51**: 299-301.

Shimoyama, T., Neelam, B., Fukuda, S., Tanaka, M., Munakata, A., and Crabtree, J.E. (1999) VacA seropositivity is not associated with the development of gastric cancer in a Japanese population. *European Journal of Gastroenterology and Hepatology* **11**: 887890.

Shiota, S., Matsunari, O., Watada, M., Hanada, K., and Yamaoka, Y. (2010) Systematic review and meta-analysis: the relationship between the *Helicobacter pylori dupA* gene and clinical outcomes. *Gut Pathogens* **2**: 13.

Skibinski, D.A.G., Genisset, C., Barone, S., and Telford, J.L. (2006) The cell-specific phenotype of the polymorphic *vacA* midregion is independent of the appearance of the cell surface receptor protein tyrosine phosphate I. *Infection and Immunity* **74**: 49-55.

Skillman, K.M., Barnard, T.J., Peterson, J.H., Ghirlando, R., and Bernstein, H.D. (2005) Efficient secretion of a folded protein domain by a monomeric bacterial autotransporter. *Molecular Microbiology* **58**: 945-958.

Smith, S.I., Kirsch, C., Oyedeji, K.S., Arigbabu, A.O., Coker, A.O., Bayerdoffer, E., and Miehlke, S. (2002) Prevalence of *Helicobacter pylori vacA*, *cagA* and *iceA* genotypes in Nigerian patients with duodenal ulcer disease. *Journal of Medical Microbiology* **51**: 851854.

Smoot, D.T., Resau, J.H., Earlington, M.H., Simpson, M., and Cover, T.L. (1996) Effects of *Helicobacter pylori* vacuolating cytotoxin on primary cultures of human gastric epithelial cells. *Gut* **39**: 795-799.

Solnick, J.V., Hansen, L.M., Salama, N.R., Boonjakuakul, J.K., and Syvanen, M. (2004) Modification of *Helicobacter pylori* outer membrane protein expression during experimental infection of rhesus macaques. *Proceedings of the National Academy of Sciences (U.S.A.)* **101**: 2106-2111.

Sommi, P., Ricci, V., Fiocca, R., Necchi, V., Romano, M., Telford, J.L., Solcia, E., and Ventura, U. (1998) Persistence of *Helicobacter pylori* VacA toxin and vacuolating potential in cultured gastric epithelial cells. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **275**: 681-688.

Soprova, Z., Sauri, A., van Ulsen, P., Tame, J.R., den Blaauwen, T., Jong, W.S., and Luirink, J. (2010) A conserved aromatic residue in the autochaperone domain of the autotransporter Hbp is critical for initiation of outer membrane translocation. *Journal of Biological Chemistry* **285**: 38224-38233.

Stein, M., Rappuoli, R., and Covacci, A. (2000) Tyrosine phosphorylation of the *Helicobacter pylori* CagA antigen after *cag*-driven host cell translocation. *Proceedings of the National Academy of Sciences (U.S.A.)* **97**: 1263-1268.

Stein, M., Bagnoli, F., Halenbeck, R., Rappuoli, R., Fantl, W.J., and Covacci, A. (2002) c-Src/Lyn kinases activate *Helicobacter pylori* CagA through tyrosine phosphorylation of the EPIYA motifs. *Molecular Microbiology* **43**: 971-980.

Stingl, K., Uhlemann Em, E.M., Deckers-Hebestreit, G., Schmid, R., Bakker, E.P., and Altendorf, K. (2001) Prolonged survival and cytoplasmic pH homeostasis of *Helicobacter pylori* at pH 1. *Infection and Immunity* **69**: 1178-1180.

Strobel, S., Bereswill, S., Balig, P., Allgaier, P., Sonntag, H., and Kist, M. (1998) Identification and analysis of a new *vacA* genotype variant of *Helicobacter pylori* in different patient groups in Germany. *Journal of Clinical Microbiology* **36**: 1285-1289.

Sundrud, M.S., Torres, V.J., Unutmaz, D., and Cover, T.L. (2004) Inhibition of primary human T cell proliferation by *Helicobacter pylori* vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proceedings of the National Academy of Sciences (U.S.A.)* **101**: 7727-7732.

Supajatura, V., Ushio, H., Wada, A., Yahiro, K., Okumura, K., Ogawa, H., Hirayama, T., and Ra, C. (2002) Cutting edge: VacA, a vacuolating cytotoxin of *Helicobacter pylori*, directly activates mast cells for migration and production of proinflammatory cytokines. *Journal of Immunology* **168**: 2603-2607.

Suzuki, J., Ohnsihi, H., Shibata, H., Wada, A., Hirayama, T., Iiri, T., Ueda, N., Kanamaru, C., Tsuchida, T., Mashima, H., Yasuda, H., and Fujita, T. (2001) Dynamin is involved in human epithelial cell vacuolation caused by the *Helicobacter pylori*-produced cytotoxin VacA. *Journal of Clinical Investigation* **107**: 363-370.

Suzuki, J., Ohnishi, H., Wada, A., Hirayama, T., Ohno, H., Ueda, N., Yasuda, H., Iiri, T., Wada, Y., Futai, M., and Mashima, H. (2003) Involvement of syntaxin 7 in human gastric epithelial cell vacuolation induced by the *Helicobacter pylori*-produced cytotoxin VacA. *Journal of Biological Chemistry* **278**: 25585-25590.

Szabò, I., Brutsche, S., Tombola, F., Moschioni, M., Satin, B., Telford, J.L., Rappuoli, R., Montecucco, C., Papini, E., and Zoratti, M. (1999) Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of *Helicobacter pylori* is required for its biological activity. *EMBO Journal* **18**: 5517-5527.

Tabassam, F.H., Graham, D.Y., and Yamaoka, Y. (2008) OipA plays a role in *Helicobacter pylori*-induced focal adhesion kinase activation and cytoskeletal re-organization. *Cellular Microbiology* **10**: 1008-1020.

Takata, T., Fujimoto, S., Anzai, K., Shirotani, T., Okada, M., Sawae, Y., and Ono, J. (1998a) Analysis of the expression of CagA and VacA and the vacuolating activity in 167 isolates from patients with either peptic ulcers or non-ulcer dyspepsia. *American Journal of Gastroenterology* **93**: 30-34.

Takata, T., Okada, M., and Ono, J. (1998b) The *vacA* genotypes in 167 isolates from Japanese patients with either peptic ulcers or non-ulcer dyspepsia. *American Journal of Gastroenterology* **93**: 482-483.

Takeshima, E., Tomimori, K., Takamatsu, R., Ishikawa, C., Kinjo, F., Hirayama, T., Fujita, J., and Mori, N. (2009) *Helicobacter pylori* VacA activates NF-*ic*B in T cells via the classical but not alternative pathway. *Helicobacter* **14**: 271-279.

Tan, S., Noto, J.M., Romero-Gallo, J., Peek, R.M., Jr., and Amieva, M.R. (2011) *Helicobacter pylori* perturbs iron trafficking in the epithelium to grow on the cell surface. *PLoS Pathogens* **7**: e1002050.

Tee, W., Lambert, J.R., and Dwyer, B. (1995) Cytotoxin production by *Helicobacter pylori* from patients with upper gastrointestinal tract diseases. *Journal of Clinical Microbiology* **35**: 1203-1205.

Telford, J.L., Ghiara, P., Dell'Orco, M., Comanducci, M., Burroni, D., Bugnoli, M., Tecce, M.F., Censini, S., Covacci, A., Xiang, Z., Papini, E., Montecucco, C., Parente, L., and Rappuoli, R. (1994) Gene structure of the *Helicobacter pylori* cytotoxin and evidence for its key role in gastric disease. *Journal of Experimental Medicine* **179**: 1653-1658.

Testerman, T.L., Conn, P.B., Mobley, H.L., and McGee, D.J. (2006) Nutritional requirements and antibiotic resistance patterns of *Helicobacter* species in chemically defined media. *Journal of Clinical Microbiology* **44**: 1650-1658.

Thoreson, A.C., Hamlet, A., Celik, J., Bystrom, M., Nystrom, S., Olbe, L., and Svennerholm, A.M. (2000) Differences in surface-exposed antigen expression between *Helicobacter pylori* strains isolated from duodenal ulcer patients and from asymptomatic subjects. *Journal of Clinical Microbiology* **38**: 3436-3441.

Tomb, J.F., White, O., Kerlavage, A.R., Clayton, R.A., Sutton, G.G., Fleischmann, R.D., Ketchum, K.A., Klenk, H.P., Gill, S., Dougherty, B.A., Nelson, K., Quackenbush, J., Zhou, L.X., Kirkness, E.F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H.G., Glodek, A., McKenney, K., Fitzegerald, L.M., Lee, N., Adams, M.D., Hickey, E.K., Berg, D.E., Gocayne, J.D., Utterback, T.R., Peterson, J.D., Kelley, J.M., Cotton, M.D., Weldman, J.M., Fujii, C., Bowman, C., Watthey, L., Wallin, E., Hayes, W.S., Weidman, J.M., Borodovsky, M., Karp, P.D., Smith, H.O., Fraser, C.M., and Venter, J.C. (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**: 539-547.

Tombola, F., Carlesso, C., Szabò, I., de Bernard, M., Reyrat, J.M., Telford, J.L., Rappuoli, R., Montecucco, C., Papini, E., and Zoratti, M. (1999a) Helicobacter pylori vacuolating cytotoxin forms anion-selective channels in planar lipid bilayers: possible implications for the mechanism of cellular vacuolation. *Biophysical Journal* **76**: 1401-1409.

Tombola, F., Oregna, F., Brutsche, S., Szabò, I., Del Giudice, G., Rappuoli, R., Montecucco, C., Papini, E., and Zoratti, M. (1999b) Inhibition of the vacuolating and anion channel activities of the VacA toxin of *Helicobacter pylori*. *FEBS Letters* **460**: 221-225.

Tombola, F., Del Giudice, G., Papini, E., and Zoratti, M. (2000) Blockers of VacA provide insights into the structure of the pore. *Biophysical Journal* **79**: 863-873.

Tombola, F., Morbiato, L., Del Giudice, G., Rappuoli, R., Zoratti, M., and Papini, E. (2001a) The *Helicobacter pylori* VacA toxin is a urea permease that promotes urea diffusion across epithelia. *Journal of Clinical Investigation* **108**: 929-937.

Tombola, F., Pagliaccia, C., Campello, S., Telford, J.L., Montecucco, C., Papini, E., and Zoratti, M. (2001b) How the loop and middle regions influence the properties of *Helicobacter pylori* VacA channels. *Biophysical Journal* **81**: 3204-3215.

Torres, L.E., Melian, K., Moreno, A., Alonso, J., Sabatier, C.A., Hernandez, M., Bermudez, L., and Rodriguez, B.L. (2009) Prevalence of *vacA*, *cagA* and *babA2* genes in Cuban *Helicobacter pylori* isolates. *World Journal of Gastroenterology* **15**: 204-210.

Torres, V.J., McClain, M.S., and Cover, T.L. (2004) Interactions between p-33 and p-55 domains of the *Helicobacter pylori* vacuolating cytotoxin (VacA). *Journal of Biological Chemistry* **279**: 2324-2331.

Torres, V.J., Ivie, S.E., McClain, M.S., and Cover, T.L. (2005) Functional properties of the p33 and p55 domains of the *Helicobacter pylori* vacuolating cytotoxin. *Journal of Biological Chemistry* **280**: 21107-21114.

Tsuda, M., Karita, M., Morshed, M.G., Okita, K., and Nakazawa, T. (1994) A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. *Infection and Immunity* **62**: 3586-3589.

Tsutsumi, R., Higashi, H., Higuchi, M., Okada, M., and Hatakeyama, M. (2003) Attenuation of *Helicobacter pylori* CagA-SHP-2 signalling by interaction between CagA and C-terminal Src kinase. *Journal of Biological Chemistry* **278**: 3664-3670.

Tsutsumi, R., Takahashi, A., Azuma, T., Higashi, H., and Hatakeyama, M. (2006) Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with *Helicobacter pylori* CagA. *Molecular and Cellular Biology* **26**: 261-276.

Tummuru, M.K., Cover, T.L., and Blaser, M.J. (1993) Cloning and expression of a high-molecular-mass major antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. *Infection and Immunity* **61**: 1799-1809.

Tummuru, M.K.R., Cover, T.L., and Blaser, M.J. (1994) Mutation of the cytotoxin-associated *cagA* gene does not affect the vacuolating cytotoxin activity of *Helicobacter pylori*. *Infection and Immunity* **62**: 2609-2613.

Unemo, M., Aspholm-Hurtig, M., Ilver, D., Bergstrom, J., Boren, T., Danielsson, D., and Teneberg, S. (2005) The sialic acid binding SabA adhesin of *Helicobacter pylori* is essential for nonopsonic activation of human neutrophils. *Journal of Biological Chemistry* **280**: 15390-15397.

Van den Brink, G.R., Tytgat, K.M., Van der Hulst, R.W., Van der Loos, C.M., Einerhand, A.W., Buller, H.A., and Dekker, J. (2000) *H. pylori* colocalises with MUC5AC in the human stomach. *Gut* **46**: 601-607.

van Doorn, L.J., Figueiredo, C., Rossau, R., Jannes, G., van Asbroeck, M., Sousa, J.C., Carneiro, F., and Quint, W.G.V. (1998a) Typing of *Helicobacter pylori vacA* gene and detection of *cagA* gene by PCR and reverse hybridization. *Journal of Clinical Microbiology* **36**: 1271-1276.

van Doorn, L.J., Figueiredo, C., Sanna, R., Pena, S., Midolo, P., Ng, E.K.W., Atherton, J.C., Blaser, M.J., and Quint, W.G.V. (1998b) Expanding allelic diversity of *Helicobacter pylori vacA*. *Journal of Clinical Microbiology* **36**: 2597-2603.

Van Doorn, L.J., Figueiredo, C., Megraud, F., Pena, S., Midolo, P., Queiroz, D.M., Carneiro, F., Vanderborght, B., Pegado, M.D., Sanna, R., De Boer, W., Schneeberger, P.M., Correa, P., Ng, E.K., Atherton, J., Blaser, M.J., and Quint, W.G. (1999) Geographic distribution of *vacA* allelic types of *Helicobacter pylori*. *Gastroenterology* **116**: 823-830. van Doorn, L.J., Henskens, Y., Nouhan, N., Verschuuren, A., Vreede, R., Herbink, P., Ponjee, G., van Krimpen, K., Blankenburg, R., Scherpenisse, J., and Quint, W. (2000) The efficacy of laboratory diagnosis of *Helicobacter pylori* infections in gastric biopsy specimens is related to bacterial density and *vacA*, *cagA*, and *iceA* genotypes. *Journal of Clinical Microbiology* **38**: 13-17.

Van Zanten, S.J., Dixon, M.F., and Lee, A. (1999) The gastric transitional zones: neglected links between gastroduodenal pathology and *Helicobacter* ecology. *Gastroenterology* **116**: 1217-1229.

Veikkola, T., and Alitalo, K. (1999) VEGFs, receptors and angiogenesis. *Seminars in Cancer Biology* **9**: 211-220.

Viala, J., Chaput, C., Boneca, I.G., Cardona, A., Girardin, S.E., Moran, A.P., Athman, R., Memet, S., Huerre, M.R., Coyle, A.J., DiStefano, P.S., Sansonetti, P.J., Labigne, A., Bertin, J., Philpott, D.J., and Ferrero, R.L. (2004) Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori cag* pathogenicity island. *Nature Immunology* **5**: 1166-1174.

Vinion-Dubiel, A.D., McClain, M.S., Czajkowsky, D.M., Iwamoto, H., Ye, D., Cao, P., Schraw, W., Szabo, G., Blanke, S.R., Shao, Z., and Cover, T.L. (1999) A dominant negative mutant of *Helicobacter pylori* vacuolating toxin (VacA) inhibits VacA-induced cell vacuolation. *Journal of Biological Chemistry* **274**: 37736-37742.

Wang, W.C., Wang, H.J., and Kuo, C.H. (2001) Two distinctive cell binding patterns by vacuolating toxin fused with glutathione S-transferase: one high-affinity m1-specific binding and the other lower-affinity binding for variant m forms. *Biochemistry* **40**: 1188711896.

Ward, D.M., Pevsner, J., Scullion, M.A., Vaughn, M., and Kaplan, J. (2000) Syntaxin 7 and VAMP-7 are soluble N-ethylmaleimide-sensitive factor attachment protein receptors

required for late endosome-lysosome and homotypic lysosome fusion in alveolar macrophages. *Molecular Biology of the Cell* **11**: 2327-2333.

Warren, J.R., and Marshall, B.J. (1983) Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* **1**: 1273-1275.

Weeks, D.L., Eskandari, S., Scott, D.R., and Sachs, G. (2000) A H+-gated urea channel: the link between *Helicobacter pylori* urease and gastric colonization. *Science* **287**: 482-485. Wen, Y., Feng, J., Scott, D.R., Marcus, E.A., and Sachs, G. (2007) The HP0165-HP0166 two-component system (ArsRS) regulates acid-induced expression of HP1186 a-carbonic anhydrase in *Helicobacter pylori* by activating the pH-dependent promoter. *Journal of Bacteriology* **189**: 2426-2434.

Willhite, D.C., Cover, T.L., and Blanke, S.R. (2003) Cellular vacuolation and mitochondrial cytochrome *c* release are independent outcomes of *Helicobacter pylori* vacuolating cytotoxin activity that are each dependent on membrane channel formation. *Journal of Biological Chemistry* **278**: 48204-48209.

Willhite, D.C., and Blanke, S.R. (2004) *Helicobacter pylori* vacuolating cytotoxin enters cells, localizes to the mitochondria, and induces mitochondrial membrane permeability changes correlated to toxin channel activity. *Cellular Microbiology* **6**: 143-154.

Wirth, H.P., Beins, M.H., Yang, M., Tham, K.T., and Blaser, M.J. (1998) Experimental infection of Mongolian gerbils with wild-type and mutant *Helicobacter pylori* strains. *Infection and Immunity* **66**: 4856-4866.

Wong, B.C., Lam, S.K., Wong, W.M., Chen, J.S., Zheng, T.T., Feng, R.E., Lai, K.C., Hu, W.H., Yuen, S.T., Leung, S.Y., Fong, D.Y., Ho, J., and Ching, C.K. (2004) *Helicobacter pylori* eradication to prevent gastric cancer in a high-risk region of China: a randomized controlled trial. *Journal of the American Medical Association* **291**: 187-194.

Wotherspoon, A.C., Ortiz-Hidalgo, C., Falzon, M.R., and Isaacson, P.G. (1991) *Helicobacter pylori*-associated gastritis and primary B-cell gastric lymphoma. *Lancet* **338**: 1175-1176.

Yahiro, K., Niidome, T., Hatakeyama, T., Aoyagi, H., Kurazono, H., Padilla, P., Wada, A., and Hirayama, T. (1997) *Helicobacter pylori* vacuolating cytotoxin binds to the 140 kDa protein in human gastric cancer cell lines, AZ-521 and AGS. *Biochemical and Biophysical Research Communications* **238**: 629-632.

Yahiro, K., Niidome, T., Kimura, M., Hatakeyama, T., Aoyagi, H., Kurazono, H., Imagawa, K., Wada, A., Moss, J., and Hirayama, T. (1999) Activation of *Helicobacter pylori* VacA toxin by alkaline or acid conditions increases its binding to a 250 kDa receptor protein-tyrosine phosphatase b. *Journal of Biological Chemistry* **274**: 36693-36699.

Yahiro, K., Wada, A., Nakayama, M., Kimura, T., Ogushi, K., Niidome, T., Aoyagi, H., Yoshino, K., Yonezawa, K., Moss, J., and Hirayama, T. (2003) Protein-tyrosine phosphatase a, RPTPa, is a *Helicobacter pylori* VacA receptor. *Journal of Biological Chemistry* **278**: 19183-19189.

Yahiro, K., Wada, A., Yamasaki, E., Nakayama, M., Nishi, Y., Hisatsune, J., Morinaga, N., Sap, J., Noda, M., Moss, J., and Hirayama, T. (2004) Essential domain of receptor

tyrosine phosphatase [3 (RPTPI3) for interaction with *Helicobacter pylori* vacuolating cytotoxin. *Journal of Biological Chemistry* **279**: 51013-51021.

Yakoob, J., Abid, S., Abbas, Z., Jafri, W., Ahmad, Z., Ahmed, R., and Islam, M. (2009) Distribution of *Helicobacter pylori* virulence markers in patients with gastroduodenal diseases in Pakistan. *BMC Gastroenterology* **9**: 87.

Yamaoka, Y., Kodama, T., Kashima, K., Graham, D.Y., and Sepulveda, A.R. (1998) Variants of the 3' region of the *cagA* gene in *Helicobacter pylori* isolates from patients with different *H. pylori*-associated diseases. *Journal of Clinical Microbiology* **36**: 2258-2263.

Yamaoka, Y., Kodama, T., Kashima, K., and Graham, D.Y. (1999) Antibody against *Helicobacter pylori* CagA and VacA and the risk for gastric cancer. *Journal of Clinical Pathology* **52**: 215-218.

Yamaoka, Y., Kwon, D.H., and Graham, D.Y. (2000a) A M(r) 34,000 proinflammatory outer membrane protein (*oipA*) of *Helicobacter pylori*. *Proceedings of the National Academy of Sciences (U.S.A.)* **97**: 7533-7538.

Yamaoka, Y., Malaty, H.M., Osato, M.S., and Graham, D.Y. (2000b) Conservation of *Helicobacter pylori* genotypes in different ethnic groups in Houston, Texas. *Journal of Infectious Diseases* **181**: 2083-2086.

Yamaoka, Y., Kikuchi, S., el-Zimaity, H.M., Gutierrez, O., Osato, M.S., and Graham, D.Y. (2002a) Importance of *Helicobacter pylori oipA* in clinical presentation, gastric inflammation, and mucosal interleukin 8 production. *Gastroenterology* **123**: 414-424.

Yamaoka, Y., Kita, M., Kodama, T., Imamura, S., Ohno, T., Sawai, N., Ishimaru, A., Imanishi, J., and Graham, D.Y. (2002b) *Helicobacter pylori* infection in mice: Role of outer membrane proteins in colonization and inflammation. *Gastroenterology* **123**: 1992-2004.

Yamaoka, Y., Souchek, J., Odenbreit, S., Haas, R., Arnqvist, A., Boren, T., Kodama, T., Osato, M.S., Gutierrez, O., Kim, J.G., and Graham, D.Y. (2002c) Discrimination between cases of duodenal ulcer and gastritis on the basis of putative virulence factors of *Helicobacter pylori*. *Journal of Clinical Microbiology* **40**: 2244-2246.

Yamaoka, Y., Ojo, O., Fujimoto, S., Odenbreit, S., Haas, R., Gutierrez, O., El-Zimaity, H.M., Reddy, R., Arnqvist, A., and Graham, D.Y. (2006) *Helicobacter pylori* outer membrane proteins and gastroduodenal disease. *Gut* **55**: 775-781.

Yamaoka, Y., Kato, M., and Asaka, M. (2008) Geographic differences in gastric cancer incidence can be explained by differences between *Helicobacter pylori* strains. *Internal Medicine* **47**: 1077-1083.

Yamasaki, E., Wada, A., Kumatori, A., Nakagawa, I., Funao, J., Nakayama, M., Hisatsune, J., Kimura, M., Moss, J., and Hirayama, T. (2006) *Helicobacter pylori* vacuolating cytotoxin induces activation of the proapoptotic proteins Bax and Bak, leading to cytochrome *c* release and cell death, independent of vacuolation. *Journal of Biological Chemistry* **281**: 11250-11259.

Yamauchi, K., Choi, I.J., Lu, H., Ogiwara, H., Graham, D.Y., and Yamaoka, Y. (2008) Regulation of IL-18 in *Helicobacter pylori* infection. *Journal of Immunology* **180**: 12071216.

Yanai, A., Maeda, S., Hikiba, Y., Shibata, W., Ohmae, T., Hirata, Y., Ogura, K., Yoshida, H., and Omata, M. (2007) Clinical relevance of *Helicobacter pylori sabA* genotype in Japanese clinical isolates. *Journal of Gastroenterology and Hepatology* **22**: 2228-2232.

Ye, D., Willhite, D.C., and Blanke, S.R. (1999) Identification of the minimal intracellular vacuolating domain of the *Helicobacter pylori* vacuolating toxin. *Journal of Biological Chemistry* **274**: 9277-9282.

Ye, D., and Blanke, S.R. (2000) Mutational analysis of the *Helicobacter pylori* vacuolating toxin amino terminus: identification of amino acids essential for cellular vacuolation. *Infection and Immunity* **68**: 4354-4357.

Yokoyama, K., Higashi, H., Ishikawa, S., Fujii, Y., Kondo, S., Kato, H., Azuma, T., Wada, A., Hirayama, T., Aburatani, H., and Hatakeyama, M. (2005) Functional antagonism between *Helicobacter pylori* CagA and vacuolating toxin VacA in control of the NFAT signaling pathway in gastric epithelial cells. *Proceedings of the National Academy of Sciences (U.S.A.)* **102**: 9661-9666.

Yu, J., Leung, W.K., Go, M.Y., Chan, M.C., To, K.F., Ng, E.K., Chan, F.K., Ling, T.K., Chung, S.C., and Sung, J.J. (2002) Relationship between *Helicobacter pylori babA2* status with gastric epithelial cell turnover and premalignant gastric lesions. *Gut* **51**: 480-484.

Zaitoun, A.M. (1995) The prevalence of lymphoid follicles in *Helicobacter pylori* associated gastritis in patients with ulcers and non-ulcer dyspepsia. *Journal of Clinical Pathology* **48**: 325-329.

Zhang, Q.B., Nakashabendi, I.M., Mokhashi, M.S., Dawodu, J.B., Gemmell, C.G., and Russell, R.I. (1996) Association of cytotoxin production and neutrophil activation by strains of *Helicobacter pylori* isolated from patients with peptic ulceration and chronic gastritis. *Gut* **38**: 841-845.

Zhou, W., Yamazaki, S., Yamakawa, A., Ohtani, M., Ito, Y., Keida, Y., Higashi, H., Hatakeyama, M., Si, J., and Azuma, T. (2004) The diversity of *vacA* and *cagA* genes of *Helicobacter pylori* in East Asia. *FEMS Immunology and Medical Microbiology* **40**: 81-87.