RELATIONSHIP OF *HELICOBACTER PYLORI* VIRULENCE TO GASTRIC PATHOBIOLOGY IN THE WESTERN CAPE

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Abstract

Relationship of *Helicobacter pylori* virulence to gastric pathobiology in the Western Cape.

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The work presented in this thesis investigated virulence in *Helicobacter pylori* isolates of the Western Cape and related this virulent fingerprint to alterations in gastric cell physiology and cell cycle events. Initially, the "virulence profile" of 109 isolates from 86 patients with a spectrum of clinically significant gastroduodenal diseases was investigated. The distribution of the vacuolating cytotoxin A (*vacA*) demonstrated that the low cytotoxic *vacA* s1b allele was found in all (100%) of the isolates from patients with peptic ulcer disease and gastric adenocarcinoma. The non-cytotoxic *vacA* s2 allele was found exclusively in patients with gastritis alone and may be an excellent negative marker. Different mid-regions of the gene, which are associated with gastric pathology, were also differentially distributed (m1 with disease and m2 with gastritis alone). These alleles did not, however, segregate absolutely with disease; 40% of patients with gastritis alone were *vacA* s1bm1. The gene *cagA* was identified in all isolates and examination of the 3'-region of this gene revealed alterations, both in length as well as in structure and potential tyrosine phosphorylation sites. This specifically identified one third of patients with gastric cancer (*cagA* 3'-long), and may be an important marker for disease. The "classically virulent" Type I strains (*vacA* s1/*cagA*$^+$) were found in 100% of both peptic ulcer disease and gastric adenocarcinoma isolates. The sensitivity for clinically significant disease of this fingerprint was 100%, but the specificity was, however, only 43%. The structure of the *cag* pathogenicity island was then examined and deletions in the 5'-region were found more consistently (~60%) in isolates from patients without clinically significant disease. These deletions were associated with the non-cytotoxic *vacA* s2m2 allele (90%). The two genotypes of the *iceA* gene were examined and although half of the isolates contained both genes, *iceA1* was identified more often in isolates from patients with gastric cancer (81%). Variants of the *iceA2* gene, which were shown to have differences in secondary and tertiary protein structure, delineated patients with gastritis alone (*iceA2C*, 62%) and peptic ulcer disease (*iceA2D*, 53%). Combination analysis of *vacA* and *iceA* status revealed statistically significant associations between disease isolates and *vacA* s1b/*iceA1*. Nevertheless, one third of isolates from patients with gastritis alone had this virulence-associated genotype. The relationship between the virulence factors and genomic heterogeneity was thereafter examined using repetitive extragenic palindromic and random-amplified polymorphic DNA-PCR. Two clusters were produced for each method. A clustering of isolates from patients with peptic ulcer disease, with the *vacA* s1m1 genotype, a short *cagA* 3'-fragment, and REP1/RAPD2 genomic fingerprints were identified. Isolates from patients with gastric adenocarcinoma and gastritis alone

clustered in a second, unrelated grouping, but tended to divide into separate sub-clusters (gastritis alone with *vacA* s2m2, and gastric adenocarcinoma with *cagA* 3'-long). These results suggest that South African isolates from the Western Cape are partially clonal in nature, and that a virulence-associated clonal grouping for peptic ulcer disease occurs in our study population. The relationship between specific genes and gastric pathology was presented in the first half of the thesis.

The second part of the study examined whether a virulence fingerprint was biologically relevant using *in vitro* model systems. We hypothesized that bacterial lipopolysaccharide was a mechanism by which "virulent" *H. pylori* strains could alter cell physiology and pathobiology. The effects of a well-characterized lipopolysaccharide (84-183) from a toxigenic *Helicobacter pylori* on the histamine secretory capacity and proliferative/apoptotic nature of the biologically important gastric neuroendocrine ECL cell were initially investigated. In contrast to lipopolysaccharide from *E. coli* which had no effect, LPS 84-183 stimulated both histamine secretion (~1.5 fold) as well as DNA synthesis (~2.5 fold) but did not cause cell death over the 24 hr experimental time period. These effects were not mediated by activation of the gastrin/CCK_2 receptor but could be inhibited by somatostatin (~100%). Lipopolysaccharide from the same bacteria also stimulated DNA synthesis (~2.2 fold) in a model ECL cell tumor system. This appeared to be mediated by activation of polyamine synthesis and could be reversed by ornithine decarboxylase inhibition (~75% inhibition). This effect appeared to be specific to *Helicobacter pylori* and involved activation of the lipopolysaccharide receptor, CD14. Inhibition of lipopolysaccharide binding to CD14 reversed DNA stimulatory effects. In addition, CD14 message appeared to be upregulated in tumor ECL cells compared to the naïve cell. The effects of 84-183 were also tested in a gastric cancer model system, the AGS cell line. DNA synthesis was stimulated (~4.5 fold), was mediated by binding to CD14, and appeared to be mostly mediated by activation of stimulatory G-proteins (calcium influx [80%] and cyclic adenosine-5'-monophosphate production [95%]). Analysis also suggested that the lipid A portion of *Helicobacter pylori* LPS may be involved in specifically activating DNA synthesis. Cell cycle progression ($S+G_2M$ and the ratio of growth to apoptosis) was stimulated by *Helicobacter pylori* lipopolysaccharide. This correlated with DNA synthesis. Finally, the effect of lipopolysaccharides from South African *Helicobacter pylori* isolates whose virulence fingerprint had been defined were examined in the AGS cell system. This indicated that organisms with the *vacA* s1bm1, an intact *cag* pathogenicity island, and a long *cagA* 3'-fragment were all associated with an increased DNA synthesis. The *iceA* genotype was not associated with any significant alterations. Cell cycle progression was stimulated by lipopolysaccharides from isolates with an intact *cag* pathogenicity island, while apoptosis was stimulated by deletions in the *cag* pathogenicity island and the *vacA* s1m2 fingerprints. Analysis of effects by disease process demonstrated that gastric adenocarcinoma isolates had the most profound stimulatory effect on DNA synthesis (~4.6 fold) and cell cycle progression and an inhibitory effect on apoptosis. The data presented in this thesis support the hypothesis that *H.*

pylori virulence plays an important but not exclusive role in the pathogenesis of clinically significant gastroduodenal diseases.

Declaration

1, Mark Simon Geoffrey Kidd, hereby declare that the work on which this thesis is based is original (except where acknowledgements indicate otherwise) and that neither the whole work nor any part thereof has been, is being, or is to be submitted for another degree to this or any other University.

Signed: Signed by candidate

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The data presented in this thesis are related to the following publications:

PAPERS

1. Kidd M, Miu K, Perez-Perez GI, Blaser M, Tang LH, Sandor A, Modlin IM. *Helicobacter pylori* lipopolysaccharide stimulates histamine release and DNA synthesis in purified rat ECL cells. **Gastroenterology** 1997; 113:1110-1117.
2. Kidd M, Modlin IM. A century of *Helicobacter pylori*: Paradigm's lost, paradigm's regained. **Digestion** 1998; 59:1-15.
3. Kidd M, Tang LH, Schmid SW, Miu K, Modlin IM. A polyamine mediated mitogenic mechanism in enterochromaffin-like cells of mastomys. **Am J Phys** 1998; 275:G370-6.
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5. Louw JA, Kidd M. *Helicobacter pylori*: Friend or foe? **Specialist Medicine** 1998; 20:6-8.
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8. Kidd M, Tang LH, Schmid S, Louw JA, Modlin IM. *Helicobacter pylori* lipopolysaccharide alters ECL cell proliferation via a CD14 receptor and polyamine pathway in mastomys. **Digestion** 2000; (in press).

ABSTRACTS

1. Miu K, Kidd M, Sandor A, Tang LH, Blaser MJ, Modlin IM. *H. pylori* lipopolysaccharide (LPS) stimulates DNA synthesis and histamine release in purified rat enterochromaffin-like (ECL) cells. **Gastroenterology** 1996; 110:A463.
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modulated in part by a novel CD14 receptor mediated polyamine pathway. **Gastroenterology** 1998; 113:A2572.

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7. Kidd M, Peek RM, Lastovica AJ, Kummer AF, Louw JA. Relationship between *Helicobacter pylori iceA* genotypes, *vacA* alleles and clinically significant disease in South Africa. **S Afr Med J** 2000; (in press).
8. Kidd M, Lastovica AJ, Perez-Perez GI, Tang LH, Modlin IM, Louw JA. The biological relevance of *Helicobacter pylori* virulence fingerprints. **S Afr Med J** 2000; (in press).

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List of Abbreviations

Base pair (bp)
Blood antigen binding (*bab*)
Campylobacter pylori (C. pylori)
Campylobacter jejuni (C. jejuni)
Cholecystokinin (CCK)
Cytotoxin associated gene (Cag/*cag*)
Enterochromaffin-like (ECL)
Flow cytometry (FCM)
Gastric adenocarcinoma (GCa)
Gastrin releasing peptide (GRP)
Growth medium-colony stimulating factor (GM-CSF)
Half-maximal inhibitory concentration (IC_{50})
Half-maximal stimulatory concentration (EC_{50})
Heat shock protein (HSP)
Helicobacter pylori (H. pylori)
Induced by contact with epithelium (ice)
Insertion sequence (IS)
Interleukin (IL)
International Agency for Research on Cancer (IARC)
Kilobase (Kb)
Kilodalton (kDa)
Lewis (Le)
Lipopolysaccharide (LPS)
Mucosa associated lymphoid tissue (MALT)
Megabase (Mb)
Non-ulcer dyspepsia (NUD)
Odds ratio (OR)
Open reading frame (ORF)
Outer membrane protein (OMP)
Pathogenicity island (PAI)
Peptic ulcer disease (PUD)
Phosphate buffered saline (PBS)
Phytohemagluttinin (PHA)
Polymerase chain reaction (PCR)
Repetitive extragenic palindrome (REP)
S-phase (DNA synthesis phase)
Somatostatin (SST)
Tetrazolium salt (MTT)
The Institute for Genomic Research (TIGR)
Transforming growth factor alpha (TGFα)
Transposase (Tnp/*tnp*)
United Kingdom (UK)
United States of America (USA)
Vacuolating cytotoxin (*vac*)
Vasoactive intestinal peptide (VIP)
World Health Organization (WHO)
Zollinger Ellison Syndrome (ZES)

Chapter 1.

Introduction and Experimental Rationale

Helicobacter pylori has dominated clinical and scientific gastroenterological research over the past two decades. Within the rubric of evolving and rapidly changing concepts, it has now become clear that the organism is the most common cause of gastritis, is associated with peptic ulcer disease, gastric lymphoma, neuroendocrine cell hyperplasia and is a candidate initiator for the development of gastric cancer in humans [1]. This is, at least, the paradigm of the developed world.

In Africa, the disparity between the occurrence of *H. pylori* infection (up to 80% prevalence) and the perceived low expression of clinically significant disease (other than gastritis) has led some to speculate that this organism does not play a major role in the etiology of upper gastrointestinal pathology in developing nations [2]. It should, however, be acknowledged that our understanding of the epidemiology of gastroduodenal diseases in the African setting, with its generally poor medical infrastructure, is less than perfect. Preliminary investigations suggest that the prevalence of gastric adenocarcinoma may be as high as 8% in some African endoscopic studies [3]. In Cape Town and environs, the prevalence of *H. pylori* infection ranges between 40% and 80% in subjects with dyspeptic symptoms, depending on the socio-economic status of the individual [4]. In addition, both the increased risk for gastric adenocarcinoma (estimated prevalence: 3%) as well as the importance of chronic atrophic gastritis as a precursor lesion for this condition has been noted in a preliminary, screening study [5].

In the West, it is accepted that, in spite of the fact that *H. pylori* infection is extremely common throughout the world, most infected people never develop ulceration or adenocarcinoma and remain asymptomatic in a milieu of long-term infection [1,6]. Development of disease is thought to depend at least on host factors as well as on the virulence of the infecting strain [6]. Like many bacterial pathogens, *H. pylori* produces and secretes proteinaceous toxins. These may play a role in *H. pylori*-mediated disease. Several non-conserved bacterial virulence factors with logical roles in pathogenesis have thus far been described (to be reviewed more fully in Chapter 4) [6]. These include the following functionally linked genes: the various alleles encoding vacuolating cytotoxin activity (*vacA*), the cytotoxin associated gene (*cag*) pathogenicity island (including *cagA* and *cagE*), the *iceA* (induced by contact with epithelium) gene family, possibly the *bab*

genes, as well as the expression of the immune invading host Lewis X (Le^x) and Y (Le^y) in the cell wall lipopolysaccharides [7-10]. Data from Western studies demonstrate that there is an association between peptic ulcer disease and *vacA* subtype s1, and the CagA protein [11]. There is also some data implicating *iceA1*, and Lewis antigen expression in gastrointestinal disease [10,12]. In addition, expression of the CagA protein has been postulated to be a marker for increased risk of gastric atrophy and gastric adenocarcinoma [13,14].

In the South African setting, however, no studies have attempted to determine systematically, whether differences in pathogenic strains are associated with alterations in gastric pathophysiology, including gastric cancer. The initial focus of this study was, therefore, to analyze, at a genetic level, possible virulence factors in *H. pylori* isolated from patients in the Western Cape with a spectrum of gastroduodenal disease (gastritis alone, peptic ulcer disease and gastric adenocarcinoma). The presence (or absence), structure, DNA sequence and predicted proteins of these factors would be analyzed and the relationship to disease type examined (Chapter 5-7). In addition, the relationship of genomic heterogeneity to virulence would be explored (Chapter 8).

H. pylori infection results in gastric mucosal endocrine and exocrine perturbations, recruitment of inflammatory cells and alterations in cellular proliferation and apoptosis (reviewed in Chapters 3 and 4). A number of *in vivo* and *in vitro* systems have been established to try and examine these alterations, and include *H. pylori* infection in rodent models, or analysis of *H. pylori* products in gastrointestinal cell lines [15-21]. Investigations of the latter kind have analyzed either the effects of bacterial products on cells over different time points or have attempted to mimic the gastric environment with co-culture experiments. Both have their limitations. Contradictory results have been obtained in these *in vitro* studies and no clear delineation of a particular effect apart from vacuolation [11] has thus far been established. Biologically relevant cells of the gastric mucosa include the following:

i) The naïve ECL cell

The neuroendocrine histamine-secreting ECL cell is the primary link between gastrin and the production of gastric acid from the fundic parietal cell and is thus a critical interface between the peripheral and central regulation of acid secretion. In addition, this cell is activated by gastrin to proliferate, and may produce and secrete growth factors, particularly histamine and TGFα, during growth [22]. Such a panoply of factors and responses may play some role in modulating gastric pathophysiology.

ii) The transformed ECL cell

A novel rodent model available for study of the neoplastic transformed ECL cell is the *Mastomys (Praomys) natalensis*. Use of acid inhibitory agents in this Southern African animal results in the rapid production of hypergastrinemia with a transformation from a normal ECL cell population to a hyperplastic state by two months and neoplastic lesions (benign neuroendocrine tumors) are evident in up to 80% of animals by four months [23,24]. The hyperplastic ECL state is reversible at 8 weeks but by 16 weeks withdrawal of the gastrin stimulus fails to result in tumor regression [23]. The use of the mastomys species has been useful in studying gastrin induced ECL cell proliferation and has enabled characterization of the pathobiology of the ECL cell tumor.

iii) Gastric (AGS) cancer cell line

The gastric AGS cell line is derived from fragments of a gastric adenocarcinoma from a fifty-four year old female Caucasian patient [25]. It has an epithelial morphology and is tumorigenic in athymic BALB/c mice. It is also one of the two gastric cell systems most used in the study of *H. pylori*.

H. pylori alters both the physiology and the pathology of the gastric mucosa, often through undefined mechanisms. The second component of the study was undertaken in order to assess whether and by what mechanism *H. pylori* could differentially affect the physiology of biologically relevant gastric cells. Investigators have thus far focused primarily on specific *H. pylori* pathogenic factors including vacuolating cytotoxin, CagA, urease and heat shock proteins. In this study, lipopolysaccharide (LPS) was chosen as the bacterial product to investigate based on a number of observations. These included that LPS has known modulatory effects on gastric physiology, has mitogenic potential as well as apoptotic activity, and has been chemically analyzed. The possibility also exists that the structure of LPS may be related to the virulence fingerprint of *H. pylori* and that it may therefore be a surrogate marker for virulence of the organism. This study initially investigated the effects of *H. pylori* LPS on naïve and transformed ECL cells (cf. Chapters 9 & 10). These observations were then expanded to include effects on the model gastric epithelial AGS cell line (cf. Chapter 11) and to test whether LPS from a range of clinical isolates differentially affected the pathobiology of this cell line (cf. Chapter 12).

This thesis thus comprises two parts: Firstly, an analysis of *H. pylori* virulence in clinical isolates and its relationship to gastroduodenal diseases *in vivo* and, secondly, an assessment of the effects of "virulence" on biologically relevant gastric cell lines *in vitro*.

Chapter 2.

The evolution of a genus

2.1 The History of Helicobacter pylori – its discovery and significance

The association between gastric infection with *Helicobacter pylori* and the presence of polymorphonuclear leukocytes was re-discovered by Robin Warren in 1979. The organism was cultured at the Royal Perth Hospital, Western Australia in 1982, and the relationship between the organism, gastritis and ulceration was suggested by Barry Marshall in 1984. The latter also provided the enthusiasm and drive to cement this link. Historically, however, investigations of the gastric mucosa are at least a hundred years old [26] and it is probable that the identification of spiral gastric organisms could have been made by a number of individuals (Table 2.1).

Table 2.1 The discovery of *H. pylori*

Date	Discovery
1875	Bacteria were discovered in the floor and margins of gastric ulcers by Bottcher and Letulle. First hypothesis that bacteria caused ulcer disease [27].
1889	"Spiral" bacteria were found in gastric aspirations by Jaworski [28].
1893	Spirochetes were noted by Bizzozero in the gastric mucosa (infiltrating gastric glands and found within the cytoplasm and vacuoles of parietal cells) of dogs [29]. These organisms were named *Helicobacter bizzozeronii* in 1996 [30].
1896	Mice successfully infected with *H. bizzozeronii* by Salomon [31].
1906	Krienitz noted spirochetes in the gastric aspiration of a stomach cancer patient [32].
1921	Edkins (the discoverer of gastrin in 1905) investigated the physiology of *Helicobacter felis* in the cat [33].
1938	The association between spirochetes and gastric inflammation was noted in the *Macacus* monkey and in man by Doenges [34].
1940	Freedberg and Barron "confirmed" that this organism had no etiologic role in gastric disease in man [35].
1940	The hypothesis that an "acidophilic bacteria" caused ulcer disease was first postulated by Gorham [35].

Table 2.1 The discovery of *H. pylori* (continued)

1979	*Campylobacter pylori* was identified as a possible causative agent of human gastritis by Warren [36].
1982	*C. pylori* was cultured by Kosras and Royce [37].
1985-87	Ingestion of *C. pylori* was demonstrated to cause gastritis in human volunteers [38,39].
1994	NIH concludes that there is a strong association between *H. pylori* and ulcer disease [http://www.cdc.gov/ncidod/dbmd/media.htm#history].
1994	The IARC, part of the WHO classifies *H. pylori* as a group I carcinogen [40].
1997	Genome of *H. pylori* released by TIGR [41].
1999	Comparison of two *H. pylori* genomes published [42]

Adapted from Kidd and Modlin, *Digestion* 1998; 59:1-15 [26]

2.2 Molecular and biochemical characteristics of H. pylori

Helicobacter pylori was the sixth prokaryotic genome to be sequenced. The genome of the organism consists of 1.64 to 1.67 Mb pairs, with approximately 1495 to 1590 predicted coding sequences [41,42] (Table 2.2).

Table 2.2 Comparative features of 2 unrelated virulent *H. pylori* genomes

	H. pylori 26695	*H. pylori* J99
Size (base pairs)	1,667,867	1,643,831
(G+C) content (%)	39	39
vacA genotype	*s1am1*	*s1bm1*
cagA genotype	Positive	Positive
Complete *IS605* copies	5	0
Partial *IS605* copies	8	5
Genes (ORFs)*	1,590	1,495
Classified	875	895
No function	275	290
Strain specific ORFs	345	367

ORFs* = open reading frames

Adapted from Alm et al. Nature 1999; 397:176-180 [42]

Features of specific importance of this genome include small size, the ability to establish a positive inside membrane potential, well-developed systems for motility, as well as a number

of specialized genetic factors, including regulation of gene expression (DNA restriction and modification), physiology and metabolism, iron acquisition and molecular mimicry [43,44]. The limitations of the new sequence databases are highlighted by the fact that approximately one-third of the 1590 predicted coding sequences from *H. pylori* 26695 were not even assigned putative identifications [41]. Furthermore, for many of the 1091 sequence identifications, a biological role appears to have been assigned only on the basis of database matches of less than 50% similarity. Nevertheless, many putative adhesins, lipoproteins, and about 300 membrane proteins have been identified which underscore the potential complexity of the host-pathogen interaction. Consistent with its restricted niche, *H. pylori* has few regulatory networks, and a limited metabolic repertoire and biosynthetic capacity [41]. The organism contains enzymes for glucose metabolism, lacks β-galactosidase, has some of the enzymes of the Kreb's cycle, and also the urease operon encoding UreA and UreB (urease). Synthesis of urease is constitutive, accounting for as much as 15% of the organism's protein and is essential for the successful colonization of the gastric mucosa [45]. The genome also encodes several outer membrane proteins (OMPs), some of which are porins, able to transport a variety of molecules into and out of the periplasmic space. The presence of homopolymeric tracts (polyC or polyG) and dinucleotide repeats (e.g. CT or AG) present in the genome suggests that *H. pylori*, like several other mucosal pathogens, uses recombination and slipped-strand mispairing within dinucleotide repeats as mechanisms for antigenic variation and adaptive evolution [41].

2.3 Other Helicobacter species

Over the past 20 years, research in mutually overlapping disciplines has demonstrated that *Helicobacter pylori* is only one member of a whole family of bacteria which infect the entire gastrointestinal tract of both humans and animals. To date, at least 7 urease positive microaerophilic *Helicobacter* species have been cultured from mammalian and avian stomachs. *H. acinomyx* is the natural cheetah gastric helicobacter, *H. mustelae* is adapted to the stomach of the ferret, and *H. nemestrinae* is found in the primate stomach. *H. felis*, *bizzozeronii, salmonis* and *heilmannii* do not have host species specificity and have been identified in a number of animal species including cats, dogs, pigs, lemurs and monkeys [46-51]. Most of these gastric helicobacters appear to cause minimal disease in their hosts [52].

Genus-specific *H. pylori* probes, based on conserved sequences within the 16S ribosomal RNA gene, have revealed a wide-range of new *Helicobacter* species in a number of animals and birds [53]. (Table 2.3).

Table 2.3 *Non-gastric helicobacters*

Helicobacter species	Host	**Urease activity**
H. bilis	Rodent	**Positive**
H. canis	Dog	**Negative**
H. cholecystus	Hamster	**Negative**
H. cinaedi	Hamster/gerbil	**Negative**
H. fennelliae	Hamster/gerbil	**Negative**
H. hepaticus	Rodent	**Positive**
H. muridarum	Rodent	**Positive**
H. pametensis	Bird/Swine	**Negative**
H. pullorum	Poultry	**Negative**
H. rodentium	Rodent	**Negative**
H. trogontum	Rodent	**Positive**

Adapted from Lee and Robertson, *Kluwer Academic Publishers*; 1998:3-12 [30]

Urease is essential for the successful colonization of the gastric mucosa [45] and is central to the pathogenesis of *H. pylori*. Only some of the lower bowel organisms are urease-positive and it appears likely that gastric helicobacters may have evolved from the urease-positive lower bowel colonizers like *H. muridarum* [30]. Some of these organisms, particularly if they translocate from their normal niche to other parts of the gastrointestinal tract, may be important in disease processes. Evidence for this is provided by *H. pullorum* which has been observed to cause inflammatory lesions in the livers of chickens, probably following translocation into the portal blood-stream and into this organ [54]. In mice, translocation of *H. hepaticus* from the lower bowel causes hepatitis which can progress to hepatocellular carcinoma in the livers of these animals [55]. Of interest are the recent findings of a hepatic *Helicobacter* species identified in patients with chronic cholecystitis [56,57]. These organisms have been suggested as possible risk factors for gall-bladder disease in populations with bile-resistant *Helicobacter* species. It appears certain that the genus of *Helicobacter* will continue expanding, and that some of these organisms will play a role in human disease.

Chapter 3.

Evidence implicating *H. pylori* as a gastric pathogen

3.1 Introduction

In 1965, Sir Austin Bradford Hill, Professor Emeritus of Medical Statistics set out a list of criteria to analyze, logically, a causal relationship between environmental agents and disease [58]. While this cannot provide a formal test of causality, it does provide the framework to generate a verdict of causation from an observed association. The variables, which are summarized in Table 3.1, are not mutually independent.

Table 3.1 Hill's outline

Variable
Strength and specificity of association
Consistency and coherence of the observed association
Biological plausibility and experimental evidence
Is there a temporal relationship?
Is a biological gradient involved?
Analogy

Infection with *H. pylori* is associated with functional effects (alterations in gastric physiology e.g. acid secretion) and epithelial effects (alterations in gastric mucosa e.g. gastritis). Hills' criteria were borne in mind when examining the evidence implicating the organism as a causative agent/gastric pathogen in this chapter.

3.2 Functional effects of H. pylori

Evidence relating *H. pylori* infection to alterations in gastric physiology includes the following:

3.2.1 Effect on gastrin

Experimental evidence and clinical observations demonstrate that gastric mucosal colonization by *H. pylori* results in increased basal serum gastrin levels and may result in increased post-prandial plasma gastrin concentrations and increased gastrin responses to stimulation with GRP compared to *H. pylori*-negative healthy volunteers [59-62]. The G-17 form of gastrin is the form selectively increased by infection, but the mechanisms responsible

for this remain unknown. One proposal for the alteration of gastrin release is the production of an alkaline microenvironment by acid-neutralizing ammonia catalyzed by the bacterial urease. This elevation of pH in the vicinity of the G-cells could potentially result in gastrin release. Although complete urease inhibition does not result in a decrease of gastrin secretion [63,64], it has been noted that long-term exposure to ammonia results in hypergastrinemia [65]. An alternative potential mechanism might be the production of gastrin-stimulating cytokines such as interleukin-1β and tumor necrosis factor-α from T-lymphocytes and monocytes present in the inflammatory infiltrate engendered by *H. pylori* [66,67]. A third possibility is an increase in gastrin release consequent upon inhibition of somatostatin (SST) synthesis and release [68]. In rare circumstances, *H. pylori* infection can result in massive hypergastrinemia to an extent that may mimic Zollinger-Ellison syndrome (ZES) [69] or the entity of antral G-cell hyperfunction [70].

3.2.2 Effect on acid secretion

The data on the effects on acid secretion are not clear-cut. Observations, in early infection, suggest that acid secretion decreases (by 1980 there was a report concerning an "epidemic gastritis associated with hypochlorhydria" [71]). During the chronic phase of infection, it can be demonstrated that duodenal ulcer patients have, on the average, higher acid output than patients without duodenal ulcer [72,73]. Counting the number of parietal cells has shown that duodenal ulcer patients have more parietal cells thus explaining the acid output data [74,75]. There is also experimental evidence for at least two acid inhibitory substances which have been purified from *H. pylori*, with one partially sequenced [76]. This appears to represent a metabolic gene.

3.2.3 Effect of site of infection

The gastric effects of *H. pylori* appear to depend in part on the site of infection. In the case of antral infection, clinical observations suggest that there is a decrease of somatostatin or the D-cell population accompanied by hypergastrinemia [77]. In the case of infection of the gastric corpus, there is often gastric atrophy with loss of the acid secreting cells; this could account for the reduced level of acid secretion observed in patients with gastric atrophy [75]. Hypergastrinemia is thought to be trophic for parietal cells, thus accounting for the higher acid output in duodenal ulcer patients.

3.3 Gastritis

In most people, gastritis is not associated with any obvious symptoms. Infection with *H. pylori*, however, is observed to result in a cellular infiltrate in the gastric mucosa termed chronic gastritis, and visible damage both to the superficial epithelial cells and to the cells that form the pits and glands of gastric mucosa [78]. Attachment of the organism also releases a sequence of inflammatory changes which results in injury and altered function both to the superficial and glandular mucosa, including the oxyntic glands [79]. Virtually all infected persons develop gastric inflammation that persists for the duration of the infection [80]. Gastritis can be antral predominant or corpus predominant, or can affect both areas of the stomach (pangastritis) [77,81]. The physical distribution of the organism is important because it may determine the physiological and pathological outcome of the gastritis although this has not been empirically proven. This is supported by evidence demonstrating that eradication of the organism leads to healing of the gastritis and the return of mucosal histopathological appearances to normal. The causal role for the organism in gastritis has been accepted [82,83]. *H. pylori* has been isolated from a patient with gastritis, purified and ingested. In both cases, a gastritis of varying degrees had developed in the human "guinea-pig" [38,39]. Koch's postulates (Table 3.2) have therefore been fulfilled for gastritis [84].

Table 3.2 Koch's postulates

First postulate	"The organism should always be found microscopically in the bodies of animals having the disease and in that disease only; it should occur in such numbers, and can be distributed in such a manner as to explain the lesions of the disease."
Second postulate	"The organism should be obtained from the diseased animal and grown outside the body"
Third postulate	"The inoculation of these organisms, in pure cultures, freed by successive transplantations from the smallest particle of matter taken from the original animal, should produce the same disease in a susceptible animal."
Fourth postulate	"The organisms should be found in the diseased areas so produced in the animal."

3.4 H. pylori and Peptic Ulcer Disease

There is substantial evidence associating *H. pylori* with peptic ulcer disease. This includes the following: *H. pylori* carriers appear to have an increased risk of developing peptic ulcer disease (about 3-50 fold) depending on the site of infection [85-88]. The organism is present in

90 to 95% of patients with duodenal ulcer and 60 to 80% of patients with gastric ulcer as compared to 25-30% incidences in asymptomatic control subjects [89-92]. More recently, a surprisingly high incidence of *H. pylori* negative patients with duodenal ulcer disease has been recorded in the USA [93]. This, however, could simply be a statistical anomaly and be a reflection of NSAID usage [94]. There is an association between gastric metaplasia in the duodenal cap and *H. pylori* which appears to confer a predisposition to ulcer disease. *H. pylori* can colonize only gastric-type epithelia, and is not therefore found normally in the duodenum. Gastric metaplasia, the replacement of the columnar cells which normally cover the duodenal villi, by gastric type epithelium rich in neutral mucin, is present in up to 90% of patients with duodenal ulceration, and appears to allow the organism to colonize the duodenal bulb [95,96]. The definitive link between the organism and this disease is provided by the unequivocal association between the eradication of *H. pylori* infection and the cure of peptic ulcer disease [97-99]..

3.5 H. pylori and Gastric cancer

Although gastric cancer is a disease with a poorly understood pathogenesis [100], some evidence links *H. pylori* with it. The effect of infection generated by the organism may result in three types of mucosal alterations: 1) The gastric mucosa regenerates; 2) an adaptive, reparative process with the replacement of the normal functioning mucosa resulting in atrophy may occur or 3) intestinal metaplasia may develop. Mucosal atrophy is thought to be the key pathological change underlying the increased risk of gastric cancer in subjects with chronic gastritis [86,101] while the progression of intestinal metaplasia to colonic type intestinal metaplasia is a known risk factor for gastric cancer [102]. If *H. pylori* is to be implicated as the starting point for a sequence of changes ultimately leading to cancer, it is necessary that the organism plays a defined role in the pathogenesis of these two processes.

3.5.1 Atrophy

Irreversible loss of functional structure or atrophy occurs when destroyed glands fail to regenerate and the space that they previously occupied in the lamina propria is replaced by fibroblasts and extracellular matrix [101]. *H. pylori* appears to play a role in atrophy. The evidence supporting this includes the following. Data from all parts of the world indicate that *H. pylori* infection is the major cause of chronic atrophic gastritis [103]. One of the modes of cell death in glandular atrophy is apoptosis. Evidence for the induction of apoptosis by *H.*

pylori has been obtained recently from two types of study, namely the identification of apoptotic cells in tissue sections from *H. pylori* infected individuals, and the induction of apoptosis in cultured gastric epithelial cells *in vitro*. Several studies by independent groups have all shown that the *H. pylori* colonized stomach contains more apoptotic cells than normal [104-106]. Furthermore, the increased number of apoptotic epithelial cells decreases to normal following eradication, suggesting that the bacterium or the associated inflammatory response is responsible for the increased apoptosis [104]. It is not known, however, whether *H. pylori* induces a physiological, senescent type of cell death in normal but aging cells, or an altruistic apoptosis in response to cell damage [107]. What is also not known, is whether the induction of apoptosis by *H. pylori* may be the stimulus for gastric mucosal proliferation [108], or whether apoptosis may be viewed as an attempt to reduce tissue growth in response to hyperproliferation growth [17]. It is not known whether apoptosis in this context is a damaging or protective effect.

Direct injury to epithelial cells by bacterial products (cytotoxins, ammonia, phospholipases and others) is likely to be important for surface epithelia but is potentially less important with regard to glandular epithelia [79]. Other likely mechanisms are host responses to infection. For *H. pylori* infection, the mucosal response to infection leans heavily towards that of the promotion of inflammation, autoantibody formation, and cell-mediated epithelial damage, all of which are classical Th1 mediated responses [79]. The link therefore between *H. pylori* and atrophy may be an indirect one that is thought to be modified by the ability of the host to mount appropriate autoimmune responses. Irrespective of the mechanisms involved, a recent study has demonstrated that eradication of the organism led to improvements in gastric atrophy in a prospective randomized study [109]. This suggests that progression of pathologic changes in the gastric mucosa can be potentially inhibited.

3.5.2 Metaplasia

In cases of intestinal metaplasia, the specialized gastric glandular epithelial cells are replaced by an intestinal type epithelium containing goblet cells, intermediate/absorptive cells which in most cases is lined by a brush border. Both mucosal atrophy and intestinal metaplasia are putative precursors of gastric cancer [110]. Once chronic changes (e.g. intestinal metaplasia) have been established in the gastric mucosa, there is no good evidence for a return of mucosa to normal (reversibility). The known association of distal gastric cancer (both the intestinal and diffuse types) with pre-existing gastritis has led a number of investigators to transfer the

association from gastritis to its cause, *H. pylori* infection [5,110,111]. Data from all parts of the world demonstrate that *H. pylori* infection is associated with the presence of intestinal metaplasia [112], although the organism colonizes adjacent areas only. At least one study has shown that eradication of the organism leads to regression of intestinal metaplasia activity over a one year study period [109].

3.5.3 Cellular role in gastric tumor formation

Development of gastric cancer is a complex and poorly understood process involving alterations in the balance of gastric mucosal proliferation and apoptosis/atrophy.

3.5.3.1 Alterations in proliferation

Recently it has been reported that *H. pylori* infection is associated with the increased proliferation of gastric epithelial cells in *H. pylori* positive hosts [113,114], and eradication of *H. pylori* significantly reduces gastric epithelial cell proliferation [113,115]. Research into *H. pylori* has focussed attention on the importance of chronic inflammation and impaired host defense mechanisms as factors in the development of gastric cancer. It is apparent that *H. pylori* colonization in the gastric mucosa causes acute inflammatory cell infiltration and leads to chronic inflammation in all cases sustaining *H. pylori* infection [116]. Following this, there are evidently differences in the severity of the inflammation of the gastric mucosa due to differences in the extent of the gastric atrophy [112]. An association between antibody titer and the subsequent development of gastric cancer has been claimed, suggesting greater extents of inflammation leading to mutagenesis and gastric cancer [87]. It is likely that *H. pylori* associated inflammation may interact with other causal factors such as environmental or hereditary factors related to gastric carcinogenesis and result in the intestinal type of early gastric cancer. Thus, at least the intestinal types of gastric carcinoma may be long-term sequelae of *H. pylori* infection.

The relationship between *H. pylori* infection and the diffuse type of gastric cancer seems to be different from that of intestinal cancer. In the case of diffuse-type gastric cancer, the sequence of *H. pylori* infection, atrophy of the gastric mucosa, intestinal metaplasia and development of gastric cancer does not seem to be relevant [102]. Instead, DNA damage due to oxygen radicals induced by persistent inflammatory cell infiltration in the gastric mucosa based on *H. pylori* infection may lead to alterations of the gene and result in the development of diffuse-type gastric cancer [117].

A number of studies have attempted to identify whether *H. pylori* or its products could directly influence either proliferation or the rate of proliferation of gastric epithelial cells. Table 3.3 and Figure 3.1 summarizes the results to date:

Table 3.3 Experimental observations analyzing the effects of *H. pylori* on cell proliferation *in vitro*.

Cell type	*H. pylori* constituent	**Method**	Mechanism	Results	Reference
AGS (gastric cancer line)	Whole organism	FCM	?	$cag^+ > cag^-$	21
AGS (gastric cancer line)	Whole organism	FCM	?	$cagA^+ = cagA^-$	20
AGS (gastric cancer line)	Whole organism	Ki-67 FCM and MTT assay	?	Increased, *H. pylori* > *E. coli*	15
MKN28 (gastric epithelial cell)	Dialyzed/ undialyzed broth filtrates	?	?	Decreased	16
IEC-6 (small intestine, epithelial)	Water extract	24 hr incubation, ^{3}H-thymidine uptake for 4 hr	Receptor mediated?	Increased, *E. coli* > *H. pylori*	18
IEC-6 (small intestine, epithelial)	Sonicates & water extracts	24 hr incubation, ^{3}H-thymidine uptake for 4 hr	Receptor mediated?	Increased, *E. coli* > *H. pylori*	19

FCM = flow cytometry; MTT = tetrazolium salt; ? = no mechanism suggested

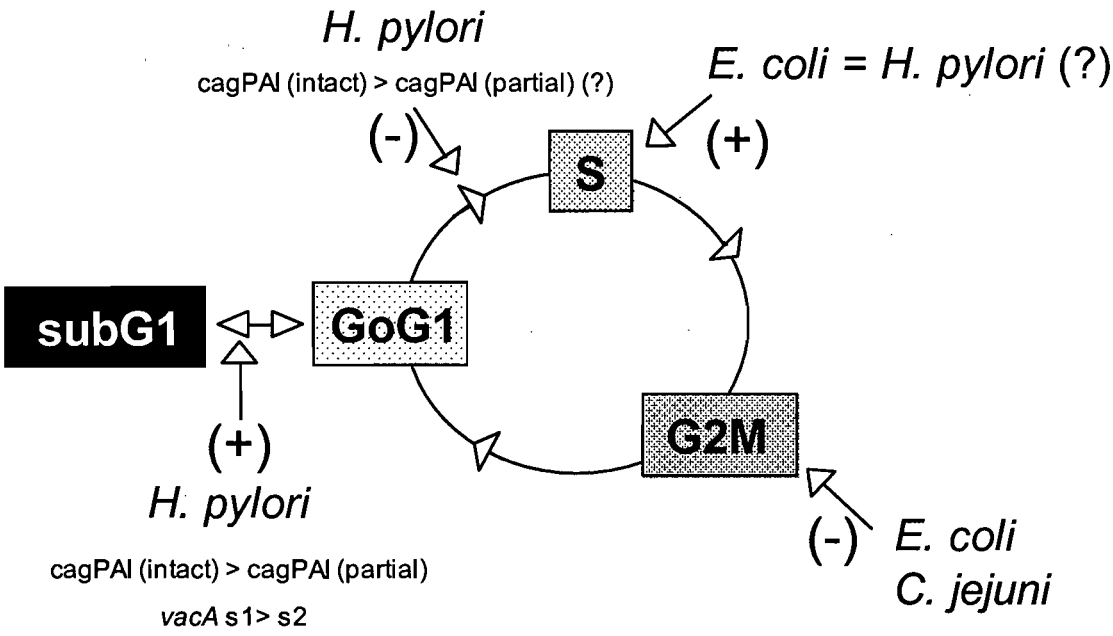


Figure 3.1 Compilation of *in vitro* data relating *H. pylori* or its products to alterations in the cell cycle. *H. pylori* (or its constituents) may inhibit entry into the S-phase or stimulate DNA synthesis and activate apoptosis (entry into sub-G_1). Other gastrointestinal organisms inhibit entry into the G_2M phase.

These results reflect the difficulties inherent in this field. Studies in gastric carcinoma cell lines demonstrate either an increase [15,21] or a decrease [16,20] in cell proliferation – discrepancies which may be due to differences in the methodologies used. The two studies analyzing the effects of products of *H. pylori* demonstrated an effect on proliferation but at levels less than that obtained from *E. coli* products [18,19]. These experiments were conducted in a small intestinal cell line, which minimizes the relevance of the findings. It is obvious that a proliferative role for *H. pylori* has not been clearly or adequately defined *in vitro*.

3.5.3.2 Alterations in apoptosis

Recent studies have shown that exposure of bacterial proteins and whole bacteria to gastric epithelial cells inhibits proliferation of gastric epithelial cells *in vitro* [17,20,76,118]. In one study, soluble bacterial protein led to a decrease in epithelial cell proliferation that was detectable within 16 hrs. [76]. In addition, protein synthesis was inhibited by exposure to bacterial proteins, which was seen initially 6 hours after exposure. The inhibitory effect was not caused by lytic activity (cell death) and was believed to be linked to a protein with an estimated

molecular weight of approx. 100 kDa. Proteins from knockout mutants without urease, the gene *cagA*, or VacA activity were shown to have the same effects [76]. In other experiments, inhibition of cell proliferation could be induced by culture with bacteria (albeit at high concentrations: >100 organisms per cell) [20], soluble extracts and culture supernatant [17]. This suggests that the factor originates from the cytosol of the organism and is released into the culture medium. In addition, this factor was sensitive to trypsin digest and heating at 95°C, suggesting that it may be a protein [17]. This protein also appeared to be present in all bacterial strains. Interestingly, this inhibitory protein appeared to be specific to *H. pylori* because *C. jejuni* was unable to affect gastric epithelial cell growth and apoptosis. A further study has suggested that $cagA^+$ organisms are less effective than $cagA^-$ organisms in inducing apoptosis [20]. It should be pointed out that while this *in vitro* data suggest that inhibition of epithelial proliferation may be a general phenomenon of all *H. pylori*, this may not directly translate to the *in vivo* scenario where it has been suggested that there are considerable strain-specific differences in gastritis and the immune response [17].

3.5.3.3 The neuroendocrine ECL cell

The mucosal cells implicated in gastric tumorigenesis are unknown. The cellular origin of tumors may be potentially interesting because such knowledge may be important in understanding tumorigenesis itself, mechanisms for preventing tumor formation and also planning therapeutic approaches. Recently, some focus has been applied to the gastric ECL cell. Neuroendocrine differentiated tumor cells in malignant tumors of the gastrointestinal tract have been known for many decades [119,120]. In tumors classified as adenocarcinomas due to a glandular growth pattern and mucin staining of malignant cells, the presence of neuroendocrine tumor cells was believed to represent dedifferentiation. The biological rationale for classification of tumors as carcinoids when the majority of the cells have a neuroendocrine marker and as adenocarcinomas when only few tumor cells have such markers remains to be empirically proven. An alternative view is that all these tumors could be of neuroendocrine origin with differing degrees of dedifferentiation. This suggests that tumors with the least neuroendocrine positive cells are the most dedifferentiated and therefore most malignant. A previous study has described neuroendocrine tumor cells of the ECL cell type in gastric carcinomas of the diffuse type, but not in tumors of the intestinal type [121]. In a more recent prospective study of gastric tumors, it has been found that 27% of tumors had cells positive for chromogranin A reactivity (a marker for endocrine cells), and five (four

diffuse type, 1 intestinal type) of the 41 carcinomas analyzed required reclassification as neuroendocrine tumors [122]. Among these tumors, the ECL cell was the most preponderant cell of origin. In addition, tumors of the diffuse type were most often reclassified as neuroendocrine carcinomas. Interestingly, it also appears that ECL cells may be implicated in the pathogenesis of spontaneous oxyntic mucosal adenocarcinomas found in inbred cotton rats [123]. A role for ECL cells in gastric carcinoma formation has not, however, been proven.

The direct effect of *H. pylori* on the pathogenesis of ECL cell proliferation is not known, but there are a number of observations which implicate the bacterium in the process of disease. Micronodular pseudohyperplasia appears to be a passive process of endocrine cell clustering, which is a frequent finding in foci of gland atrophy occurring in *H. pylori* gastritis [124-126]. To date, the phenomenon of endocrine cell clustering appears to be restricted to patients with *H. pylori* colonization but has not been demonstrated to progress beyond micronodular hyperplasia, which only occasionally has been of the pre-dysplastic adenomatoid type [125-127]. Whether these phenomena are due to a direct mitogenic effect of the bacterium or are the result of perturbations in gastrin secretion is not known. It is of interest to note, however, that the effects of *H. pylori* are more marked in fundic mucosal gastritis than in antral gastritis. ECL cell hyperplasia and the potential for neoplastic formation in a setting of *H. pylori* induced gastritis remain enigmatic.

3.5.4 Experimental gastric adenocarcinoma in animal models

The carcinogenicity of *H. pylori* has been proven in the Mongolian gerbil [128]. The animal (*Meriones unguiculatus*) is an appropriate animal in which various gastrointestinal diseases which mimic human *H. pylori* infection have been studied [129-131]. This animal model can be successfully infected with *H. pylori* and short-term infection with Tox^+ (toxic, $VacA^+/CagA^+$) strains can result in severe gastritis, gastric ulceration and intestinal metaplasia. The long-term effects of infection in this model (62 weeks) result in the formation of pyloric adenocarcinomas in a third of infected animals [128]. The tumors originate deep in the gastric gland, but the progenitor cell type has not yet been identified. Significant changes consistent with chronic atrophic gastritis were also noted in the oxyntic mucosa of these achlorhydric animals [128]. In addition, neuroendocrine ECL cell tumors, diagnosed by the presence of neuroendocrine nests were identified in 1 in 10 animals. Extending such studies to 80-100 weeks demonstrated increases in serum gastrin levels, the increased prevalence of gastric carcinoids as well as well-differentiated intestinal-type gastric cancers [132,133]. Such results

fulfil Koch's postulates in these animals and also suggest strongly the link between *H. pylori* infection and both carcinoid and carcinoma in humans [134].

3.5.5 H. pylori and Gastric MALT Lymphoma

Mucosa-associated lymphoid tissue (MALT) in the stomach is primarily the consequence of an infection with *H. pylori* [135]. Other agents may also result in gastric MALT, but it is believed that in more than 90% of the cases, infection with *H. pylori* is responsible for the induction of lymphoid tissue in the stomach. MALT is an immunologic defense system to control local infection caused by the organism. It is composed of *H. pylori*-reactive T cells, plasma cells, and other B-cells and mimics lymphoid follicles known from typical nodal tissue. Localization of MALT parallels the site of *H. pylori* infection, the antrum [136,137]. In addition, the number of lymphoid follicles in the stomach correlates well with the grade of *H. pylori*-induced inflammation [135].

In 1991, data emerged to indicate that low-grade gastric MALT lymphomas are a result of genetic changes probably induced in B-cells clonally evolving from *H. pylori*-related chronic gastritis [138]. Lymphomas arising from gastric MALT show specific features not present in other lymphoma entities. They arise from the marginal zone of the lymphoid follicle, they consist of centrocyte-like cells, and lympho-epithelial lesions must be present to establish the diagnosis of gastric MALT lymphoma [139,140].

Wotherspoon et al. have observed that microscopically detectable MALT lymphomas associated with *H. pylori* infection responded to eradication of *H. pylori* [140]. Similar findings were reported by Rudolph [141], and Stolte [135] (Table 3.4). Roggero et al. recently stated that the cure of *H. pylori* infection in low-grade gastric MALT lymphoma should be considered standard therapy [142].

Table 3.4 Summary of the remission induction in Low-Grade gastric MALT lymphomas using cure of *H. pylori* infection.

N	CR (%)*	Year	Reference
10	60	1992	[135]
6	83	1993	[140]
33	69	1995	[143]
25	60	1995	[144]
15	93	1996	[145]
84	80	1997	[146]

CR = complete remission.

Adapted from Thiede et al. Gastroenterol 1997; 113:S61-S64 [146]

Since cure of *H. pylori* infection appears to be effective in low-grade gastric MALT lymphomas, some authors have speculated whether high grade lymphomas might respond also to such treatment. Only sporadic cases of cure have been published [141,147]; this is not currently the recommended approach.

3.5.6 Cohort studies

The strongest evidence that *H. pylori* plays a causal role in gastric adenocarcinoma stems from 3 sero-epidemiological case-control studies, nested within cohorts. These studies reported odds ratios (OR) between 2.8 and 6.0 [85-87] (Table 3.5). Another 4 nested case-control studies have since yielded supporting [148] and less convincing results [149-151]. In a more recent case-control study, which attempted to assess and control potential confounding bias by socio-economic status, an increased risk of non-cardia gastric cancer was observed for *H. pylori* seropositive individuals compared with seronegative subjects [152]. This association was stronger in the antrum (OR = 6.94) and particularly the pyloric region (OR = 15.1), then in the fundus (OR = 3.76) and body (OR = 2.91). The overall risk in this study was 2.26. A statistically significant negative association between *H. pylori* seropositivity and subsequent adenocarcinoma development, however, was noted in the cardia. This latter finding suggests that cardia and non-cardia cancers may be regarded as separate disease entities with different risk profiles.

Table 3.5 Studies assessing *H. pylori* antibody positivity & cancer risk

Patient	Control	OR*	Region	Reference
20/29 (69%)	54/116 (47%)	2.8	UK	85
92/109 (84%)	72/109 (66%)	3.6	USA	86
103/109 (94%)	83/109 (76%)	6.0	Hawaii	87
166/208 (80%)	619/983 (63%)	2.26	Norway	152

OR* = Odds Ratio

H. pylori infection is also implicated as a risk factor for diffuse gastric cancer. A recent nested case-control study from Norway has demonstrated that the OR for both intestinal (OR = 4.1) and diffuse cancer (OR = 5.18) was equally strong [152].

In 1994, after exhaustively reviewing the literature, the Working Group Meeting of the International Agency for Research on Cancer held in Lyons, in affiliation with the World Health Organization, concluded that there was sufficient evidence from human studies to establish the carcinogenicity of infection with *H. pylori* and that *H. pylori* infection is carcinogenic to humans (group 1 carcinogen) [40]. As mentioned, some studies support this position, while others have produced equivocal results which may engender some doubts.

3.5.7 Epidemiological studies

Indirect evidence in support of a relationship between *H. pylori* infection and gastric cancer is provided by the following epidemiological observations. During the early part of this century, gastric cancer was the most common malignant neoplasm in the USA and in European countries, but since then rates (particularly of distal cancer) have progressively declined in parallel with the decline in the prevalence of *H. pylori* [153]. Data from the Netherlands show that since 1950, there has been an approximately 50% decrease in the incidence of distal gastric cancer which is associated with a decrease in *H. pylori* prevalence [154]. Interestingly, as there has been a decrease in distal gastric cancers, there has also been an increase in the incidence of proximal gastric cancers [100]. This increase has also been related to differences in *H. pylori* prevalence [155]; in the presence of *H. pylori*, development of adenocarcinoma of the cardia is in fact lower than in *H. pylori* negative subjects [152]. In South Africa, preliminary investigations suggest that stomach cancer may be ranked as high as 7th in males (making up 3.4% of all cancers) [156], and some endoscopic studies suggest that the age-standardized risk

of gastric adenocarcinoma in Cape-colored males may be as high as 20.41/10,000 while the lifetime risk is 1 in 40.5 people [3]. It should be acknowledged, however, that our understanding of the epidemiology of gastroduodenal diseases is less than perfect.

3.6 H. pylori and gastroduodenal pathology in Africa

The relationship between *H. pylori* infection and gastric pathology in Africa is controversial. Several authors have suggested that there is no correlation between the organism and any pathology apart from gastritis [2,157], and that infection with the organism may indeed be protective against *inter alia* gastric cancer [158]. The evidence for these views is derived largely from sero-epidemiology studies in "asymptomatic" individuals combined with anecdotal evidence about the prevalence of various disease entities [2,157-160]. More recently, the results of a case-control study suggest that there may be no differences in the infection rates between patients with gastric cancer and asymptomatic, matched controls in South Africa [161]. These observations allow for, amongst others, a hypothesis including a role for *H. pylori* related virulence factors in determining the outcome of infection.

A retrospective literature review of all the data published on *H. pylori* in Africa provide information that is at odds with time honored wisdom [3]. This review questioned the assumptions regarding gastroduodenal disease in Africa. The difficulty with definitively ascribing a pathogenic role to *H. pylori* in the African setting, is potentiated by a generally poor medical infrastructure, and aggravated by a paucity of appropriate studies directly investigating this. What seems to be apparent is that incidences of disease quoted in the 1990s are based on information gathered twenty to thirty years ago [159,160]. A critical evaluation of the data suggested that the organism's association with gastritis might be as strong as in the rest of the world. In addition, although flawed, data generated in the endoscopy era appear to suggest that infection is strongly associated with peptic ulcer disease, and that this disease itself may not be as rare as is commonly perceived (ulceration was noted in 26% of cases) [3]. There also appeared to be a preponderance of duodenal ulcer disease compared to gastric ulcer disease in these studies, and also findings of a relatively high prevalence of gastric adenocarcinoma (range 1-7%). This review suggests that the relationship between *H. pylori* and gastroduodenal disease in Africa may be very similar to that of developed countries, with the caveat that prospective, endoscopic studies are clearly needed. It is possible that a perceived low prevalence of *H. pylori* associated diseases in the setting of a high *H. pylori* carrier rate, may possibly be another expression of Africa's poor medical infrastructure, as

evidenced by the "health for all indicators" of the World Health Organization's 1996 Report [162]. It is clear that the average life expectancy in Sub-Saharan Africa is much lower than in industrialized countries, the 1995 life expectancy being 53 years (range 40 to 71). This is of particular importance in diseases such as gastric cancer which typically involves the older patient.

3.5 Conclusion

This chapter has used Hill's criteria as a basis for evaluating the strength of evidence supporting the role of *H. pylori* as a gastric pathogen.

In summary, the association between the organism and a host of endocrine and paracrine alterations suggests strongly that *H. pylori* affects gastric physiology, although direct experimental evidence is lacking. Nevertheless, a strong, consistent, and coherent association implicating *H. pylori* exists. For the entity of gastritis, associations are found in numerous studies and demonstrate strength, consistency, and specificity of association, as well as providing a biologically plausible, coherent causal relationship. The data for a causal relationship with peptic ulcer disease are supported by evidence of both specific and coherent observations and a biologically plausible relationship has been defined. Some questions, however, still relate to the strength and the consistency of an association. There is some experimental evidence linking the organism with neuroendocrine tumor formation in a susceptible animal model, but a strong and consistent association has not been proven in man. The summation of the data amassed on the relationship between *H. pylori* and gastric cancer cover associations with atrophy and intestinal metaplasia, epidemiological and cohort studies, and experimental evidence. It is clear, using Hill's criteria, that the data support a causative role for the organism. The fact that all individuals infected with *H. pylori* do not develop gastric cancer suggests strongly that modulatory factors (diet, host factors, differences in virulence) must, however, exist. A causal relationship between infection and clinically significant disease in Africa is proven for gastritis. It probably exists for peptic ulcer disease and gastric cancer in certain populations, but more research is required on this continent.

Chapter 4.

H. pylori and factors associated with disease expression

4.1 Introduction

Since its rediscovery and culture in 1982, *H. pylori* has been investigated at the genetic, metabolic and protein levels. *H .pylori* is a complex organism, which displays heterogeneity both at a genomic and a cellular level. The relationship between infection with the organism and gastrointestinal disease is obviously complex and multifactorial. Universal factors (including flagella, urease) allow the organism to colonize and evade host defences while a number of factors differentially expressed by the organism (*vacA*, *cag*PAI, *iceA*) may modulate the disease process. This chapter will outline some of those factors which may identify organisms that are "virulent" as well as indicate how such factors may potentially be relevant to the etiopathology of the disease processes.

4.2 H. pylori heterogeneity – a potentially misleading observation?

H. pylori appears to be genetically one of the most diverse bacterial species so far reported [163], although the methodology used to arrive at these conclusions is limited by its dependency on either the nucleotide sequence or on the sequencing of specific loci in different strains [163,164]. In spite of methodological limitations, the organism appears to be subject to the highest known rate of intraspecific bacterial recombination [165]. The panmictic structure of the *H. pylori* genome has been demonstrated [166], as has the possibility that it is clonal over short time periods after natural transmission [165] and there is evidence for a different "Asian" clonal grouping [167]. It has been suggested that such diversity may reflect the bacteria's ancient ancestry, its niche in varied human populations, its large numbers in colonized hosts, its ability to mutate over decades of colonization within a single host and the ease with which it may exchange its genes with other *H. pylori* strains [163,168].

An alternative viewpoint is presented by genomic-sequence comparison. A recent report has suggested that the overall genomic organization, gene order and predicted proteins of two unrelated virulent *H. pylori* isolates may be quite similar [42]. DNA–sequence differences (approx. 20%) between these two virulent strains were found mainly in the third position of coding triplets (a site for synonymous substitutions - and the reason for the enormous diversity noted in earlier studies [42,165]). This nucleotide variation does not necessarily translate into a highly divergent proteome; comparisons of *H. pylori* 26695 and

J99 demonstrated that whilst only eight genes had greater than 98% nucleotide identity, 310 proteins (41 of which had perfect identity) had 98% amino-acid conservation [42]. In addition, such a genome comparison also indicated a low level of gene shuffling; 85% of genes appear to have the same neighbor. Such a conservation of gene strings suggests little evolutionary divergence between these two virulent strains. This study, however, reported the presence of strain-specific plasticity zones (analogous to the pathogenicity islands) which contain almost half of the genes unique to each of the organisms and which may be important in the disease process [42].

Several genotyping studies have suggested that *H. pylori* may cluster in disease-specific strains. Duodenal ulcer strains appear to be more homologous to each other than to strains from patients with gastritis [169], while cluster analysis of repetitive extragenic palindromic (REP) fingerprints has demonstrated that duodenal ulcer isolates, as a group, may be more similar to one another and different from gastritis isolates in patients from the USA [170].

4.3 Strain heterogeneity

A number of techniques demonstrate that while there appears to be considerable inter-patient *H. pylori* genomic variability [164,171-177], intra-patient variability ranges from non-existent [172,178-180] to marked [171,180,181]. Multiple strain colonization with *H. pylori* may be important in determining both clinical outcome of infection and the response to antibiotic therapy. Intra-patient *H. pylori* genomic variability has been demonstrated by the co-existence of nitroimidazole-sensitive and resistant strains in infected patients [182,183] and by the demonstration of mixed infections with strains showing differences in the virulence-associated genes vacuolating cytotoxin gene A (*vacA*) and cytotoxin-associated gene A (*cagA*) [184]. The latter may be important as the distribution of these virulence factors, particularly the presence of the *vacA* s1 genotype and the gene *cagA*, has been implicated both in peptic ulceration [6,7,185] and gastric adenocarcinoma [13,14], while combinational differences in virulence factors have been postulated to play a role in the infection process [186].

4.4 Pathogenic factors

While certain factors appear to predispose the host to infection by *H. pylori* [187], the bacteria clearly possess a well-defined battery of pathogenic factors which allows the organism to: (1)

colonize the gastric mucosa (*iceA* gene product, urease, flagella, adhesins, acid inhibitory protein, iron-acquisition protein and heat shock proteins); (2) evade host defense (by shedding surface proteins, superoxide dismutase, catalase and poorly reactive lipopolysaccharide); and (3) damage host tissue (proteinaceous toxins). These factors allow *H. pylori* to colonize and persist in the host, thus establishing a chronic infection.

Many species produce and secrete proteinaceous toxins which play an important role in the pathogenesis of numerous infectious diseases. These represent a heterogeneous class of proteins that have evolved over millions of years for the specialized purpose of interacting with eukaryotic cells [188]. Differences in strain pathogenicity can also be achieved by the inheritance of a pathogenicity associated island (PAI), a large fragment of "alien" DNA which encodes virulence associated proteins [186].

In 1988, epithelial cell vacuolation was noted following *in vitro* exposure to *H. pylori* culture supernatant [189]. The effect was induced by 55% of tested strains and initial characterization suggested that it was caused by a high molecular weight protein. Following these early observations, this vacuolating cytotoxin (Vac A) has been extensively researched with regard to its structure, its effect on epithelial cells, and its clinical relevance.

A second protein, which is non-toxigenic and immunodominant, is CagA. This protein, which is encoded by a pathogenicity island, evokes a strong systemic antibody response in the human host [190]. Such a response readily identifies infection with cag^+ strains. The identification of VacA, and strains with a pathogenicity island (cag^+) led initially to the grouping of clinical isolates into two broad families, type I and type II [191]. Type I strains expressed CagA and VacA, exhibited vacuolating activity and caused gastroduodenal disease in animal models [192]. Type II strains do not express CagA or VacA and are not associated with disease. Such a classification, however, appears to be too generalized because it excludes other potential pathogenic information including alterations and/or deletions in specific genes of the *cag*PAI (e.g. *cagE*, *cag10*), *iceA* genes as well as genes in the recently identified plasticity zone. A more focussed approach encompassing these candidate virulence genes, may suggest that markers are not fully independent of each other and, importantly, are not absolutes but may reflect degrees of risk.

4.4.1 Vacuolating Cytotoxin A (vacA)

The *vacA* gene (HP0887 in strain 26695 and JHP in strain J99) is present in all *H. pylori* strains, but the toxin is secreted in detectable amounts in only about half of the isolates. *vacA*

alleles are mosaics of any combination of signal sequence type (s1a, s1b, s1c or s2) and mid-region (m1, m1x or m2) [8]. Initial studies in the USA demonstrated that infection with strains with the *vacA* s1a genotype was linked to gastric inflammation, duodenal ulceration, *in vitro* cytotoxin activity and the presence of the cytotoxin-associated gene, *cagA*, whilst *vacA* s2 strains appear to have the least pathogenic potential [11]. When data from non-oriental world-wide studies are combined (Table 4.2), *vacA* s1 strains are also associated with peptic ulcer disease [11,185,193-196]. The link between the ability of a strain to induce epithelial vacuolation *in vitro* and peptic ulceration *in vivo* is significant but does not appear to be invariable [6].

Table 4.2 World-wide relationships between *vacA* signal sequence (s) subtypes in different disease groups [11,185,193-196].

Disease	No. of patients	*vacAs1*	*vacAs2*
NUD	305	64%	36%
PUD	616	87%	13%
GCa	143	98%	2%

NUD = non-ulcer dyspepsia; PUD = peptic ulcer disease, GCa = gastric adenocarcinoma

Studies from the Orient (China, Japan, and Korea) are somewhat at odds with this data, and suggest that in these regions, *vacA* may not as readily predict clinical outcome [197-200]. These findings have led some to suggest that geographic differences in allele expression may be mistaken for disease-specific associations [201]. Such objections, however, appear to be premature, and are limited by assuming that disease markers are fully independent of each other, when they could reflect degrees of risk. Alternatively, there is evidence to suggest that Asian strains differ from "non-Asian" strains and may have evolved as a separate clonal population [167].

Investigations of the VacA protein have demonstrated that VacA is derived from a large-molecular-size polypeptide (139 kDa) that is processed at both the N-terminus (traditional signal sequence) and at the C-terminus (approx. 50 kDa) [202]. The remaining subunit of approx. 87 kDa assembles into six copies to form the holotoxin [203]. The toxin protein is not homologous to other known proteins and acts to form vacuoles within its target cells [203]. *In vitro* studies show that the toxin is well suited to the gastric environment since it

is irreversibly activated by acid exposure [204]. The purified toxin binds specifically to, and is then internalized by, cultured epithelial cells [205]. It then acts on an undetermined target in the cytosol to interfere with membrane trafficking [206], resulting in the formation of post-endosomal compartments (or vacuoles) which have both endosomal and lysosomal features [207]. Primary cultures of human gastric epithelial cells, when exposed to bacterial broth culture supernatant containing vacuolating cytotoxin activity, appear to be more sensitive to the vacuolating activity than established tumor cell lines such as AGS and Hela cells. Hela cells themselves do not appear to have a receptor for the subtype m2 allele and hence are not vacuolized by *vacA* toxins with this allele (Figure 4.1) [208].

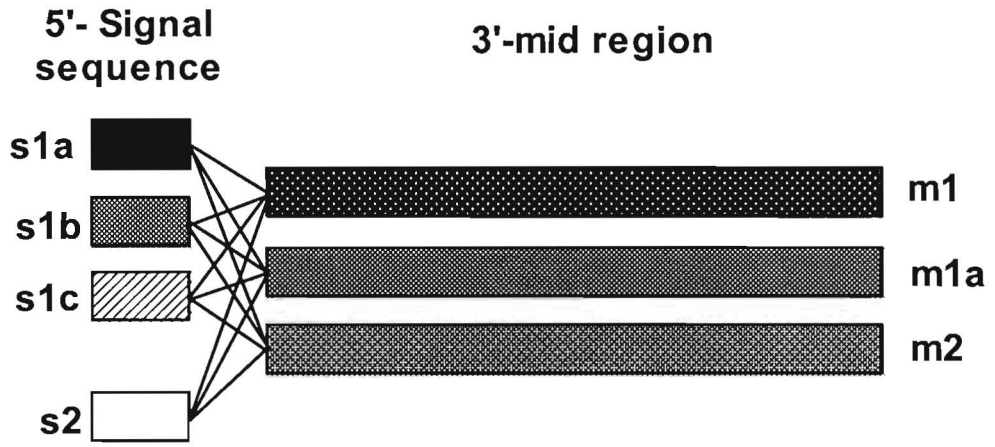


Figure 4.1 Structure of the *vacA* gene. The *vacA* s1a/s1b/s1c encodes a protein which is Tox^+ (cytotoxic). The *vacA* s2 product is non-toxic. The major *vacA* homology is present in the 3'-region (m1, m1a or m2) of the gene. Twelve protein combinations are possible. (adapted from Atherton et al. J Biol Chem 1997; 270:17771-17777 [8]).

4.4.2 cag Pathogenicity Island and cagA

The *cag* pathogenicity associated island (PAI) is a region of approx. 40 kb associated with pathogenicity and encodes a functional bacterial type IV secretion system to export CagA, and induce interleukin IL-8, NF-κB activation, c-*fos* and c-*jun* induction in *H. pylori* [176,209-213]. The island has a significantly lower concentration of guanine and cytosine residues (35%) than the rest of the genome which indicates that it was acquired from a secondary source [176,186]. It is possible that this island may be present in as much as 60-70% of organisms world-wide but this has not been systematically investigated in all populations [7,202]. It has been postulated that sub-populations of organisms with differences in virulence may have been generated following insertion of the designated insertion sequence *605*

(*IS605*), and rearrangements and deletions within the PAI [176] but this also appears not to be a universal phenomenon. The *cag* PAI can be contiguous, separated into two distinct units (*cag*I and *cag*II) by an intervening chromosomal sequence of differing length, or consist of units with partial or complete deletion or inversions (Figure 4.2).

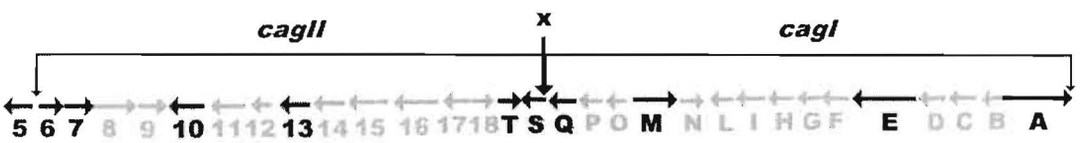


Figure 4.2 Schematic representation of the *cag*PAI. It may be divided into two sections (*cagII* and *cagI*) by an insertion sequences (*IS605*) (x). Genes in *cag*II are labeled from *cag6* to *cagS* while genes in *cag*I are labeled from *cagQ* to *cagA* (Adapted from Tomb et al. Nature 1997; 388:539-547 [41]).

*cag*II encodes 15 ORFS including *cag*6,10,S and T. Inactivation of the genes *cag*6, 10 (the *virD4* homolog), S and T (the *virB7* homolog) differentially alter IL-8 secretion [214,215]. Disruptions in genes of *cag*II are also associated with alterations in CagA phosphorylation and translocation [215]. *cagT* also has a low level of similarity to the 42 kDa IPAC surface antigen of *Shigella flexneri* [176], and is a useful marker for the presence of *cag*II or a contiguous PAI [216].

*cag*I encodes 15 open reading frames (ORFs) of different transcriptional polarity. Within *cag*I, the gene *cagA* (HP0547 in strain 26695 and JHP495 in strain J99) encodes a high-molecular-weight antigenic protein (120 - 140 kDa) [7,202]. Variability in the 3'-coding region of the *cagA* gene, which has been postulated to alter the immunogenicity of the protein, results in the differences in CagA size. The function of CagA has recently been elucidated [210-212]. Attachment of *H. pylori* results in the translocation and phosphorylation of CagA, with the resultant activation of signal transduction pathways (IL-8 secretion, NF-κB activation, c-*fos* and c-*jun* induction) [210,213]. Its production is also a useful marker for the presence, but not the integrity, of the *cag*PAI. The majority of clinical studies have focussed on expression of the gene product (i.e. CagA), rather than the gene (i.e. *cagA*), *per se*. Such clinical studies have demonstrated that CagA-producing strains occur more frequently in patients with peptic ulceration than in patients with chronic gastritis alone [7,202,217], while recent evidence has shown an association between infection with $CagA^+$ strains and the development both of atrophic gastritis and of adenocarcinoma of the stomach [13,14]. In

addition, alterations in the 3'-region of the *cagA* gene and its protein appear to correlate with clinical outcome *in vivo* [193,218]. Non-clinical studies have suggested a potential role for CagA in regulating mucosal proliferation. A recombinant fusion protein of *cagA* (containing an immunodominant region of *cagA*) has been demonstrated to specifically inhibit phytohemagglutinin-A (PHA)-driven T-cell proliferation [219]. In addition, this protein was also able to inhibit the growth of GM-CSF stimulated THP-1 monocytes and Kato-3 epithelial cells. However, the growth of Hela cells, even in the presence of growth medium-colony stimulating factor (GM-CSF) was enhanced by this protein. These results suggest that the CagA antigen may be anti-proliferative but the modulatory effect of this protein is dependent on the cell type.

Upstream of *cagA* are a number of genes associated with IL-8 secretion [209,214]. The gene *cagE* appears to be critical for the induction of *in vitro* IL-8 release. Strains with a deletion in this gene lose the ability to induce IL-8 release [209]. The gene *cagE* is similar to the *virB4* gene of *Agrobacterium tumefaciens* [176].

The insertion sequence *IS605*, is an intervening sequence which separates the *cag*PAI into *cag*II and *cag*I [176,220]. It is sometimes present between the genes *cagQ* (similar to the *Neisseria gonorrhoeae* prepilin peptidase) and *cagS* (similar to the *Erwinia chrysantemi* IIABC component PTS system PTBA [176]). *IS605* encodes two putative transposases, transposase A (TnpA) and transposase B (TnpB), and full length or partial copies of this insertion sequence may also be present elsewhere in the chromosome [176]. TnpA has a high level of similarity to *IS200* from *E. coli*, while TnpB has a high level of similarity to thermophilic bacterium *PS3* gene for the transposase-like protein D38778 [176]. *IS606* is a second *H. pylori* insertion sequence. It has the same arrangement of ORFs and 95% DNA sequence identity to *IS605* [41]. Both sequences are present as discrete units in about one-third of *H. pylori* strains [220].

An intact, complete, *cag* PAI is highly associated with duodenal ulceration in *H. pylori* isolates from France and Japan [216,221], although patients with disease may harbor strains with partial or complete deletions of the *cag* PAI which has been found in 6% of French isolates. The *cag*PAI is not, however, restricted to strains causing severe gastroduodenal disease; 53% of French, 90% of United Kingdom and 82% of Japanese NUD isolates also have intact *cag*PAIs [216,221,222]. It is possible that the structure of the *cag* PAI may exhibit geographical differences. Although similar numbers of French (89%), UK (90%)

and Japanese (94%) isolates have intact *cag*PAIs, significantly more ($p < 0.05$) UK isolates have the *IS605* element (62% vs. 32% [Japan] and 17% [France]) [216,221,222].

4.4.3 iceA

A novel gene has been discovered recently, designated *iceA* (induced by contact with epithelium) [12,223]. There are two main allelic variants of the gene: *iceA1* and *iceA2*. Functionally, the *iceA1* gene is a homolog of adenine methylase [12]. *In vitro*, *iceA1* (HP1209 in strain 26695) causes increased mucosal concentrations of IL-8 [223], and the expression of the gene is up-regulated on contact between *H. pylori* and human epithelial cells [12]. Sequence analysis has demonstrated that this is a 127 amino acid protein, but in-frame alterations may result in putative proteins of 47-130 amino acids [224]. *iceA1* may be associated with peptic ulcer disease [185,223] but this is probably limited to non-Oriental countries [201].

iceA2 (JHP1132 in strain J99) has no homology to known proteins, and occurs in a number of forms based on alterations in repeat structures [224]. In its most common form, *iceA2* could encode a protein of 59 amino acids which can be divided into two conserved outer domains of 14 and 10 amino acids that flank 3 internal peptide domains of 13, 16, and 6 amino acids respectively (Figure 4.3) [225]. The 13 and 6 amino acid domains are highly conserved, but the 16 amino acid domain exists in one of two variants, 2B or 2C. Sequence analysis of multiple *H. pylori iceA2* strains has recently revealed that this internal 35 amino acid cassette may be absent or repeated resulting in deduced proteins of 24, 59, 94 or129 amino acids [224]. Five distinct *iceA2* subtypes have thus far been defined; subtypes 2B/C appear to be transcribed more readily than 2D [224].

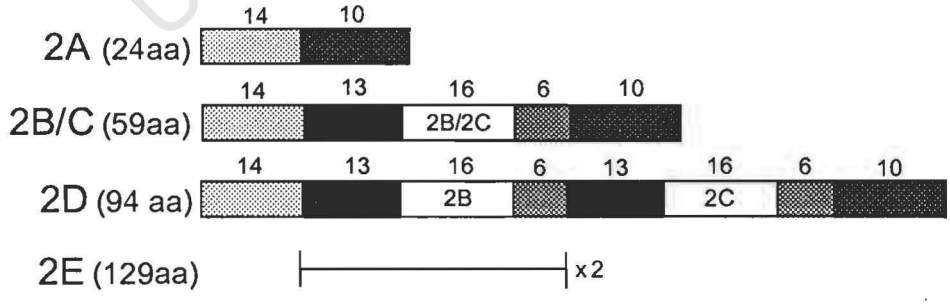


Figure 4.3 Schematic representation of the *iceA2* genotypes. The top figure represents *iceA2A*, the middle figure variants 2B and 2C, while the lower figures represents the form of *iceA2* with an additional 13-16-6 repeat. The motif in 2D is typified by 16_{2B}-16_{2C}, which is repeated twice in 2E.

4.4.4 Urease

On culturing *H. pylori*, Marshall and his colleagues initially thought the bacterium was urease negative [37]. It quickly became apparent, however, that an extraordinarily high amount of urease, up to 10-15% [226] of total cell protein was produced by the organism [227]. Numerous studies have established that urease is central to the pathogenesis of *H. pylori* since production of the enzyme is essential for successful colonization of the gastric mucosa [45].

Urease (urea aminohydrolase EC 3.5.1.5) catalyzes the hydrolysis of urea to ammonia and carbamate; the latter decomposing to carbonic acid and ammonia, with the net result of raising gastric pH. Since *H. pylori* is not acidophilic, and is sensitive to low pH (similarly to most enteric bacteria), it has been demonstrated that urease, whose pH optimum for activity ranges from 2 to 6, serves to protect the bacterium, at least *in vitro*, through local acid neutralization [228].

Seven genes are recognized to comprise the *ure* gene cluster of *H. pylori*. *UreA* and *ureB* encode the structural subunits of the enzyme itself. The nucleotide sequences of these genes predict subunits of 26.5 and 60.3 kDa, respectively [229,230]. *UreE, ureF, ureG* and *ureD* are considered accessory genes encoding proteins that function to insert nickel ions, which are required for catalytic activity. The role of *ureI* which is not essential for urease activity and does not have apparent homologues in other urease gene clusters [231] is essential for *H. pylori* survival *in vivo* and for colonization [232]. The gene product, UreI, functions as an H^+-gated urea channel regulating cytoplasmic urease [233]. Using gel filtration chromatography, the native urease has been estimated to be 550 kDa in size and to contain equimolar ratios of the UreA and UreB subunits [227]. It is generally accepted that there are six copies of each subunit in the holoenzyme, and two nickel ions fill each active site of which there are 6, located within the UreB subunit.

In contrast to other bacterial species, where ureases are entirely cytoplasmic, *H. pylori* urease can be found both inside the cytoplasmic compartment and outside adhering to the surface of the organism [234,235]. The mechanism for urease extrusion appears to be through autolysis [236]. A number of elegant *in vitro* studies have demonstrated a second function for urease, and that is the maintenance of the proton motive force which is required by the organism to survive within an acidic medium [228,237]. It would appear that at pH levels less than 6.5, the internal bacterial urease is vital for both acid protection and bacterial growth, while it has been suggested that the external urease is responsible for initiating autolysis at

pH levels close to neutral [228]. Indeed, internal urease activity is critical in allowing the organism to metabolize between pH 2.5 and 8 [237].

One of the products of urease may also play some role in mucosal damage. Direct injury to the gastric epithelial cells occurs *in vitro* from ammonia [238,239]. In animal studies, ammonia has been shown to accelerate the cell kinetics of the gastric mucosa, and long-term exposure leads to mucosal atrophy [114]. *In vitro* studies have shown that ammonia exposure to gastric cancer cell lines caused a dose-dependent delay in cell cycle progression by initiating an accumulation of cells in the S-phase of the cell cycle [240,241]. An increase in the numbers of DNA synthesizing cells, *in vivo*, may result in the generation of mutations which can lead to cancers [242]. An essential role for urease in gastric colonization has been confirmed using isogenic mutants in the nude mouse model [243]. Studies also have shown that isogenic mutant *H. pylori* strains without urease activity will not colonize gnotobiotic pigs, even when acid secretion is suppressed [244,245]. These studies show the critical role of the urease enzyme in the pathogenic effect of this bacteria and suggest also that the ammonia produced may be a nitrogen source for the synthesis of proteins necessary for bacterial adhesion.

4.4.5 Flagella

H. pylori produces polar sheathed flagella, which are believed to be essential for the bacterial colonization of the gastric mucosa. The flagella of *H. pylori* are constructed of repeating protein units (flagellin) having a molecular weight ranging from 50-62 kDa [246-248]. The nucleotide sequence of the flagellin gene *flaA* has an open reading frame of 1530 nucleotides, encoding a protein with a predicted molecular mass of 53.2 kDa. Sequence alignment of this with other flagellins demonstrates a high degree of similarity in the amino-terminal and carboxy-terminal regions, but little homology in the central domain [249]. This is supported by the fact that the flagella of *H. pylori* are antigenically related to the flagella of *C. jejuni* [248], and also to the flagellar antigens of a diverse range of gastrointestinal spiral bacteria [247]. This suggests convergent evolution, a selective advantage for this structure for gastrointestinal mucus motility. The flagella of *H. pylori* are sheathed in membranes which contain lipopolysaccharides (LPS) and proteins [250]. Flagella appear also to be important as organs of primary attachment.

4.4.6 Heat Shock Proteins (HSPs)

HSPs play a role in the pathogenesis and immunology of *H. pylori* infection. As in other bacteria, *H. pylori* has a bicistron encoding GroES-GroEL homologues. In *H. pylori*, these proteins were designated HspA (the Hsp10 homologue) and HspB (the Hsp60 homologue) [251,252]. While the HspB protein is very similar to the bacterial and eukaryotic Hsp60 chaperonins, a unique structure has been found in the HspA antigen. The N-terminal domain corresponds to the conventional Hsp10 bacterial chaperonin, but the C-terminal domain, that other bacterial homologs lack, is a nickel-binding domain. The presence of this highly charged protrusion suggests an essential role of the HspA chaperonin for the assembly or the stabilization of the urease/nickel complex in an acid environment. HspB is highly immunogenic in infected patients while only 30% of patients develop antibodies to HspA [253]. Interestingly, however, HspA has been successfully shown to protect against *H. pylori* infection in the *H. felis* mouse model [253,254].

4.4.7 Lipopolysaccharides

Bacterial endotoxins exhibit manifold and diverse biological properties and activities. In higher organisms, they elicit a broad spectrum of harmful effects that contribute to the pathogenic potential of the bacteria. The chronic, persistent infection associated with *H. pylori*, however, has suggested to many investigators that the organism has evolved to minimize its immunological response [255]. *H. pylori*, when observed by electron microscopy, has the typical trilaminar cell envelope of Gram-negative bacteria, of which the outermost layer is the outer membrane [256]. This membrane consists of a lipid bilayer, and the outermost leaflet of the lipid bilayer is composed of lipopolysaccharide (LPS). Inserted into this lipid bilayer are outer membrane proteins. Porin proteins embedded in this membrane act as channels to allow molecules to migrate into the cell, and urease is associated with the cell surface.

The general structure of LPS is a glycolipid composed of a core lipid, lipid A, and a polysaccharide region, containing the O-specific chain. Generally, the immunomodulating and immunostimulating component of LPS is provided by lipid A [257]. Structural comparisons of *H. pylori* lipid A reveal that it has less phosphate groups and fewer fatty acids than *E. coli* lipid A [258]. The length of fatty acid chain is, however, increased in *H. pylori* and additional heterogeneity has been generated by fatty acid and phosphate substitutions [258]. This heterogeneity may be reflected by altered biological activity.

The O-specific side chain is a polysaccharide composed of repeating units of a certain number of sugars, which is linked to lipid A. Of clinical interest is that these repeating units in some strains of *H. pylori* mimic the Lewis antigens expressed on erythrocytes and in the gastric mucosa. Some strains express Lewis^x, other strains express Lewis^y, whilst a third group express both [259,260]. It is possible that this mimicry may induce autoantibodies which could cause pathology in the gastric mucosa. Additionally, cross-linking of CD15 by *H. pylori* induced anti-Lewis^x antibodies may contribute to neutrophil recruitment [255].

Attachment of *H. pylori* to the gastric mucosa is mediated by blood group antigens expressed on the gastric epithelium [261]. Corresponding adhesins must therefore be present on the *H. pylori* cell surface. Such adhesins have been identified as the Lewis^x antigens [259,262], similar to the human blood group antigen which is expressed on gastric epithelial cells. Interestingly, purification of bacterial α1,3-fucosyltransferase and β1,4-galactosyltransferase and elucidation of their biophysiology demonstrated that these were the same as those used in mammals to generate Lewis^x [263]. Le^x is actively secreted into the culture medium [263] and it has been suggested that it may ultimately enter the gastric tissue [262]. The Le^x structure mediates attachment of *H. pylori* to the gastric epithelial cells at specific sites with reciprocal blood group antigens. Le^x on *H. pylori* initiates an immune response in the host [262] and may therefore explain the high prevalence of *H. pylori* induced autoantibodies which cross react with the human gastric mucosa in infected patients [264]. This autoimmune mechanism may lead to cell injury and ultimately to the development of gastritis. *H. pylori* Le^x may also be involved in the down-regulation of the T-cell immune response encountered in infected patients [262]. This activity, together with the low pro-inflammatory activity of *H. pylori* lipopolysaccharide compared to that from *Enterobacteriaceae* may ensure the survival and persistence of the organism in the stomach.

The biological activity of *H. pylori* LPS has been reported in a series of studies ranging from neutrophil interleukin-8 induction to LPS-LPS binding protein (LPB) binding studies [257]. Collectively, these results demonstrate a proportionately lower ability of *H. pylori* LPS to activate monocytes compared to LPS from other organisms. The bacterial activity of LPS is transduced via interaction with an LPS binding receptor. The best studied of these receptors is CD14, a 55 kDa glycoprotein expressed on the surface of monocytes, macrophages, neutrophils and tracheal epithelial cells [265-269]. Activation of this receptor has been associated with release or synthesis of cytokines, interleukins, oxygen radicals and the

tracheal-specific antibiotic peptide [265-269]. These responses are modulated by either tyrosine kinase activity or redistribution of intracellular G-proteins [269].

Two studies have identified a putative role for *H. pylori* LPS as a pathogenic factor in gastric disease. Such studies have demonstrated that intragastric administration of *H. pylori* LPS at doses ranging from 50 to 200 μg in rats results in stomach mucosal inflammatory responses typical of gastritis. Infiltration of the lamina propria with lymphocytes and plasma cells, edema, hyperemia and hemorrhage extending from the lamina propria to the surface mucosa, were noted in these experiments. Such changes were associated also with marked increases in epithelial apoptotic indices both in the superficial epithelium as well as deeper in the glands [270,271]. These results suggest that LPS may be a pathogenic factor responsible for the induction of gastric epithelial cell apoptosis by *H. pylori*. It should be noted that the dosage administered to the rats was high, in the order of 1-5 x 10^{-4} M, which complicates the physiological interpretation of these observations. In addition, no studies were performed on LPS from other bacterial species.

In a second series of investigations, conducted with guinea pig stomachs in Ussing chambers, *H. pylori* LPS at high concentrations (0.25mg/ml) selectively stimulated gastric mucosal pepsinogen secretion in comparison to *E. coli* LPS at the same concentration [272]. This release was not due to chief cell damage by LPS. Later studies suggested that this effect may be virulence dependent; *H. pylori* LPS mediated pepsinogen release was higher from duodenal ulcer patients compared to non-ulcer subjects [272].

The apparent LPS concentration to which the human gastric mucosa may be exposed *in vivo* is not known.

4.4.8 babA

The blood-group antigen-binding adhesin (BabA) mediates adherence of *H. pylori* to human Lewis^b blood group antigens on gastric epithelial cells [261,273]. It is a 78 kDA protein [273], and recently, the two genes encoding BabA have been cloned [9]. Only the *babA2* gene, which differs by the addition of a 10 bp insert, is functionally active [9]. The clinical relevance of this gene has been suggested in one recent study [274].

4.4.9 Lipase and Protease

Lipase and protease secreted by *H. pylori* can degrade gastric mucus, shown by the loss of protective qualities of mucus [275]. *H. pylori* protease activity leads to the disintegration of the

polymeric structure of mucin, whereas elaborated lipases and phospholipase A2 in particular result in mucous lipid degradation, loss of the mucosal surface hydrophobicity and lysophospholipid generation [275-277]. The lytic activity of the resulting lysophospholipids is detrimental to mucous gel integrity and to gastric epithelial cell membranes. It is possible that *H. pylori* has the ability to disrupt the phospholipid-rich layer at the apical surface of the mucous cells through its phospholipase activity [278-280].

Chapter 5.

Analysis of vacuolating cytotoxin A heterogeneity in *Helicobacter pylori* and its relationship to gastroduodenal disease

5.1 Introduction

The structure and function of *vacA* has been described in Chapter 4.4.1. Results from studies conducted in developed countries suggest that development of clinically significant disease is associated with virulent strains. These "pathogenic" strains are known as type I (cag^+/vac^+) as opposed to the non or low-cytotoxic type II strains [191], and have the ability to produce vacuolating cytotoxin activity *in vitro*, export CagA, and activate neutrophils directly [6,7,215,281].

The link between the ability of a strain to induce epithelial vacuolation *in vitro* and peptic ulceration *in vivo* is significant but not invariable [6]. In the USA infection with strains with the *vacA* s1a genotype has been linked to gastric inflammation and duodenal ulceration, whilst *vacA* s2 strains appear to have the least pathogenic potential [11]. We postulated that specific alleles of *vacA*, as found in Western populations, would be associated with diseases in the African setting.

5.2 Material and Methods

5.2.1 Patients, biopsy sampling and cultivation of H. pylori strains

All human studies have been approved by the Ethics and Research Committee (University of Cape Town). All patients gave informed consent for the procedure. One hundred and nine *H. pylori* isolates from 86 infected patients undergoing upper gastrointestinal endoscopy were used in this study. Seventy-two percent of the patients were Cape-colored, 20% were black and 8% were white, with a predominant male sex ratio of 70%, and a median age of forty-seven years (range: 22-63 years). Sixty-four patients had a single strain isolated from the antrum, 21 patients had strains from two anatomical sites (antrum and fundus) and one patient had three strains (duodenum, antrum and fundus). Endoscopic and histological diagnoses were recorded for all patients. In thirty-nine of the patients, no endoscopic pathology was evident, but histology confirmed the presence of chronic active gastritis, twenty-eight patients had duodenal ulcers and nineteen had gastric adenocarcinoma. Gastric biopsies obtained from the antrum of all patients were cultured as previously described [99].

Briefly, biopsies were transported in a jar under micro-aerobic conditions (Oxoid Gas Generating BR38, Basingstoke, Hampshire, UK). The specimen was cultured on tryptose blood agar (CM 233; Oxoid Ltd.) containing lysed horse blood (10% vol/vol) at 37 °C in a micro-aerobic atmosphere (12% CO2, 88% air (6% CO2), 95% humidity) for a minimum of seven days. *H. pylori* were identified by colony morphology and positive urease reaction (Christensen's urea slope). The strains were numbered, and all analyses were performed without prior knowledge of the clinical diagnosis. Reference strains 26695 [41] and J99 [42] were used in this study.

5.2.2 Preparation of samples for PCR amplification

Genomic DNA was isolated from each colony utilizing the High PCR (polymerase chain reaction) purification kit from Roche Diagnostics (Johannesburg, South Africa) [282]. Briefly, bacterial cells were resuspended in PBS (phosphate buffered saline), and lysozyme (10 mg/ml) added. Thereafter, cells were further digested by proteinase K and the DNA was isolated/purified by chaotropic salt/glass fiber methodology. Approximately 10 ug DNA was obtained from 10^6 bacterial cells.

5.2.3 PCR amplification and detection of amplified DNA products

For *vacA*, primers VA1F and VA1R were used to amplify the signal sequence [8]. Amplification fragments of 259 bp for genotype s1 and 286 bp for genotype s2 were expected (Table 5.1). Further analysis of the s1 genotype was performed using SS1F and VA1R for s1a and SS3F and VA1R for s1b. The *vacA* mid region was amplified with primers VAGF and VAGR, resulting in fragments of 570 bp for m1 and 645 bp for m2 respectively [8].

Table 5.1 Primers used to identify *vacA* alleles

Region	*Allele*	*Primer*	**Primer Sequence**	**Size (bp)**	Ref
Mid-region	m1 or m2	VAG-F VAG-R	5'-CAA TCT GTC CAA TCA AGC CAG-3' 5'-GCG TCA AAA TAA TTC CAA GG-3'	m1 = 570 m2 = 645	8
Signal sequence	s1 or s2	VA1-F VA1-R	5'-ATG GAA ATA CAA CAA ACA CAC-3' 5'-CTG CTT GAA TGC GCC AAC-3'	s1 = 259 s2 = 286	8
	s1a s1b s1c	SS1-F* SS3-F* S1C-F*	5'-GTC AGC ATC ACA CCG CAA C-3' 5'-AGC GCC ATA CCG CAA GAG-3' 5'-CTY GCT TTA GTR GGG YTA-3'	s1a = 190 s1b = 187 s1c = 213	8,201

* Used in combination with primer VA1-R

PCR amplification of *vacA* was performed as previously described [8]. Briefly, samples were incubated in Tris-reaction buffer including $MgCl_2$ [15mM], nucleotides (200uM), primers (0.2uM) and Taq polymerase as per the manufacturer's protocol (Roche Diagnostics), and subjected to PCR. Following initial denaturation, 94 °C for 1 min, each reaction consisted of 35 cycles of denaturation at 94 °C for 1 min., annealing and extension for 2 min. and final extension at 72 °C for 10 min. Annealing temperatures were set at 55 °C for VA1F, VA1R, S1C-F, VAGF, VAGR, and 59 °C for VA1R, SS1F and SS3F. Twenty microliters of each PCR mixture was subjected to gel electrophoresis on 1% agarose gels and a 100 bp DNA ladder (Roche Diagnostics) was used as a size marker.

5.2.4 Statistical methods

Data from non-identical strains (n = 109) were analyzed using the chi-squared test or Fisher's exact test as appropriate. For multiple regression, the data were summarized into two-way tables. Each table had 109 rows and columns for *vacA* genotype and disease status. The presence or absence of each character was binarily coded, present = 1, absent = 0. All analyses were performed with STATISTICA © software (Gaithersburg, Maryland, USA). Probability levels of < 0.05 were considered statistically significant.

5.3 Results

5.3.1 Determination of vacA genotypes

The gene *vacA* was detectable in all *H. pylori* isolates. By using the primers VA1F and VA1R to amplify the *vacA* signal sequences, the predicted PCR product of 259 bp for genotype s1 was obtained from eighty-nine (82%) of one hundred and nine strains as well as the two reference strains. Twenty (18%) of the strains yielded the 286 bp amplicon (s2). None of the strains yielded a PCR product of any other size. Further analysis of the eighty-nine strains with the s1 genotype revealed that 88 (99%) were *vacA* s1b and 1 (1%) was *vacA* s1a. No isolates had the *vacA* s1c allele. Strain 26695 was *vacA* s1a while J99 was *vacA* s1b.

All 109 *H. pylori* strains and the two reference strains contained DNA which was amplifiable by VAGF and VAGR, resulting in fragments of 570 bp (m1) in 73 (67%) [and in 26695 and J99] and 645 bp (m2) in 36 (33%) respectively. None had DNA amplified by both sets of primers or gave products of sizes other than predicted. Among the one hundred and nine isolates studied, 5 of the 6 possible combinations of *vacA* signal and mid regions were identified. The s1a/m1 combination was found in one (1%), s1b/m1 in 69 (63%), s1b/m2 in

18 (17%), s2/m1 in 3 (3%), and s2/m2 in 17 (17%). The s1a/m2 combination was not detected.

5.3.2 Relationship of vacA genotypes to gastrointestinal disease

All patients with peptic ulceration were infected with *vacA* s1 strains (36, 100%), compared with only 27 (57%) of 47 strains from subjects with gastritis alone ($p < 0.00001$) (Figure 5.1).

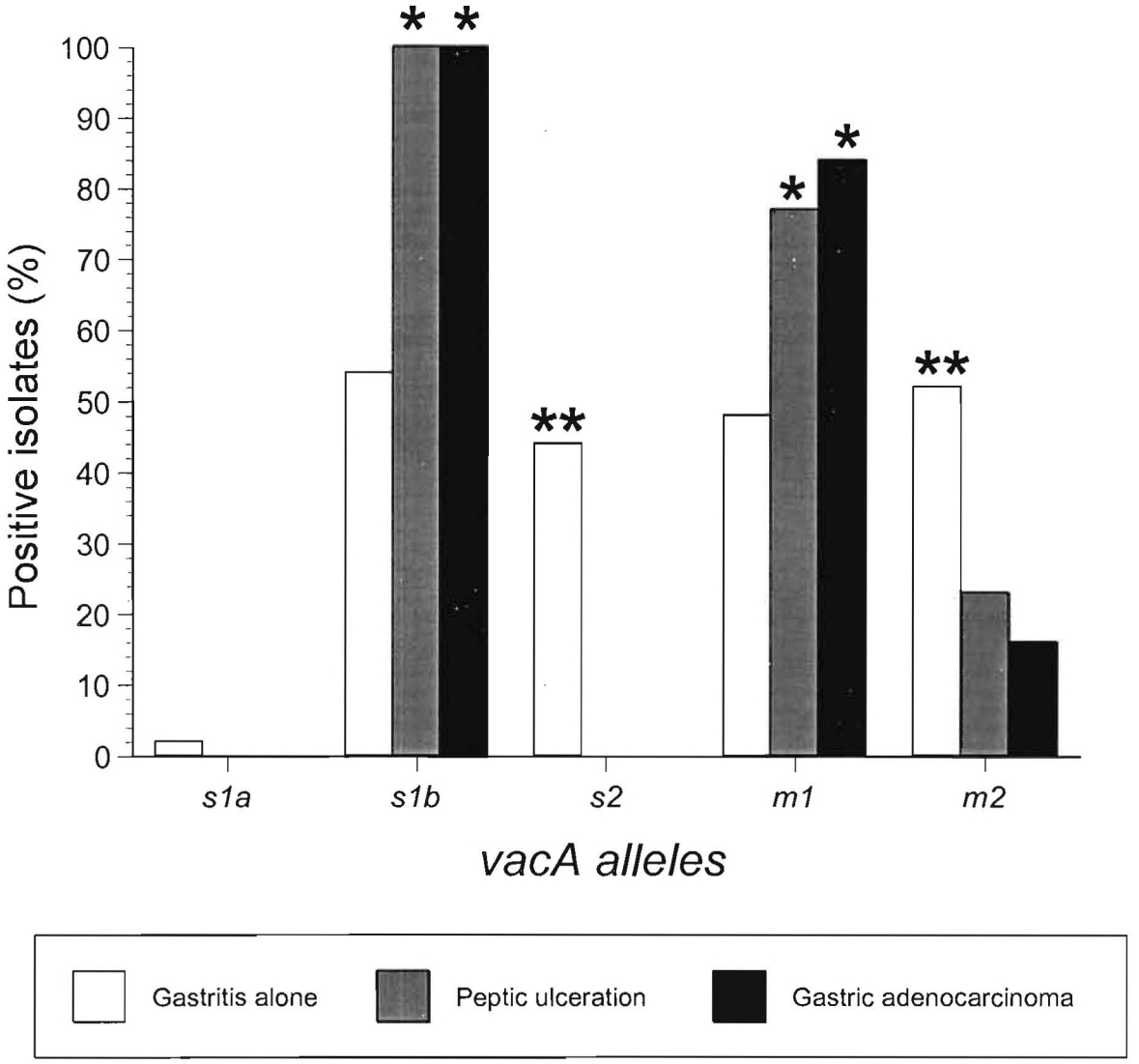


Figure 5.1 Relationship between *vacA* alleles and disease states. *$p < 0.05$ vs. gastritis, **$p < 0.05$ vs. PUD or GCa.

All of the 26 strains (100%) isolated from patients with gastric cancer were s1b ($p = 0.0002$). The *vacA* s1b/m1 genotype was found more often in strains from patients with peptic

ulceration (83%, p = 0.0007) and was more commonly associated with strains from gastric adenocarcinoma (76%, p < 0.003) than gastritis alone (40%). The strong relationship between the *vacA* s1 allele and disease was confirmed by regression analysis (Table 5.2). In contrast, the *vacA* s2 genotype was isolated exclusively in strains from patients with gastritis alone (43%), and was not found in strains from patients with either peptic ulceration (0%) or gastric adenocarcinoma (0%) (both p < 0.0002 vs. gastritis alone). In addition, the *vacA* s2m2 genotype was found exclusively in strains from patients with gastritis alone (36%). This was significantly higher than in strains either from patients with peptic ulceration (0%, p < 0.0001) or gastric adenocarcinoma (0%, p < 0.001).

Analysis of the *vacA* midregion allele types demonstrated that they were not similarly distributed in patients with gastroduodenal disease. The *vacA* m1 genotype was, however, found more often in strains from patients with peptic ulceration (83%, p < 0.001) and gastric adenocarcinoma (77%, p < 0.02) than in strains from gastritis alone (49%). Regression analysis demonstrated a statistically significant relationship between *vacA* m1 alleles and disease in these isolates (Table 5.2)

Table 5.2 Relationship between *vacA* alleles and clinically significant (both PUD and GCa) disease

Allele	r	R^2	*p-value*	β
vacA s1	0.469	0.22	*< 0.0001*	+0.469*
vacA m1	0.238	0.057	*0.049*	+0.238*

PUD = peptic ulcer disease, GCa = gastric adenocarcinoma

* = significant β

5.3.3 Predictive value of vacA s1 strains

The sensitivity of *vacA* s1 as a marker for gastric disease (either GCa or PUD) is 100% based on the data generated in this study (Table 5.3), while the specificity of *vacA* s1 as a marker is 43%. The predictive values for these strains range between 49-57%.

Table 5.3 Predictive value of *vacA* s1 allele for clinically significant disease

Diagnosis	PPV	NPV	SE	SP
PUD	0.57	1.0	1.0	0.43
GCa	0.49	1.0	1.0	0.43

PUD = peptic ulcer disease, GCa = gastric adenocarcinoma

PPV = positive predictive value, NPV = negative predictive value, SE = sensitivity, SP = specificity.

5.4 Discussion

In the present study, the results demonstrate a high prevalence of the *vacA* s1b allele and confirm the low prevalence of the *vacA* s1a allele previously found in a smaller sample [283]. Analysis of disease groups demonstrated that the *vacA* s1b allele was found consistently (100%) in strains from patients with peptic ulcer disease. In contrast to strains isolated from patients with peptic ulcer disease in the USA which are predominantly *vacA* s1a [6], no *vacA* s1a alleles were present in any South African strains associated with the disease. In the USA, *vacA* s1b strains appeared to be intermediately ulcerogenic and *vacA* alleles with this signal sequence were found to be less cytotoxic *in vitro* than *vacA* s1a strains [8,11]. South African strains with the *vacA* s1b allele tend to produce low levels of *in vitro* vacuolating activity in cultured epithelial (Hela) cells [284]. Our findings could be interpreted as being compatible with, and perhaps supportive of the "African enigma" concept. As has been noted, however, the assumption on which the "low prevalence" of disease in Africa is based can be questioned [3,5,285,286]. In addition, a high prevalence of *vacA* s1b is not limited to South Africa; *vacA* s1b alleles are also highly prevalent in Brazil [195] and Portugal [194], where reported prevalences of peptic ulceration and gastric adenocarcinoma appear to be higher. In the present study, the *vacA* s2 allele was found exclusively in the group of individuals with no endoscopic pathology other than gastritis, in keeping with observations suggesting that *vacA* s2 strains are minimally or non-ulcerogenic and potentially non-pathogenic [6,8,11].

The *vacA* middle region type also tended to be associated with the occurrence of peptic ulcer disease. Significantly more ulcer isolates had the *vacA* m1 mid-region whilst the m2 region was found more often in patients with gastritis alone. This is in keeping with a previous study which demonstrated that the m1 region was more strongly correlated with epithelial injury (epithelial degeneration, mucus depletion and microscopic erosions) than mid-region m2 [11].

The association between *vacA* genotypes and gastric adenocarcinoma has not previously been studied in the African setting. Twenty of the 26 strains in the patients in this study were *vacA* genotype s1b/m1. All of the strains were *vacA* s1b^{+}. One may hypothesize that infection with *vacA* m1 strains, and the associated potential damage to the antral and corpus mucosa may play a role in the etiology of gastric adenocarcinoma in our study population.

The temptation of over-interpreting these results is recognized. Both peptic ulcer disease and gastric adenocarcinoma are multifactorial diseases, and it is probable that factors other than the virulence of the organism plays a role in the etiology of the disease. The findings would support the postulate, however, that disease is more likely to be associated with specific *H. pylori* strains. It is apparent that these studies are limited by the timing of invasive clinical investigations and, furthermore, that it is only those organisms present in the gastric milieu at the time of biopsy which have been analyzed. The resultant is that our data can only demonstrate that at a particular time in the natural history of infection, certain strains are more likely to be associated with pathology.

5.5 Conclusion

The high prevalence of *vacA* s1b alleles in *H. pylori* strains isolated from South African individuals with peptic ulceration and gastric cancer has been noted. While, in general, attention has been focussed on the positive correlation with disease, the segregation of *vacA* s2 alleles with "non-pathogenic" strains may be an excellent negative marker.

These studies suggest that a number of the associations established between *H. pylori* virulence factors and disease in other parts of the world apply to the South African setting. Cytotoxic strains (*vacA* s1m1) are associated with disease while less cytotoxic strains (*vacA* s2m2) are found exclusively in patients with gastritis alone. There are, however, some interesting differences. The prevalence of *vacA* s1b is extremely high, while that of the highly cytotoxic *vacA* subtype 1a is very low. The widespread prevalence of a low cytotoxic *vacA* s1b allele, however, may have important ramifications and requires further study.

Chapter 6.

Analysis of the cytotoxin associated gene pathogenicity island of *Helicobacter pylori* in relationship to gastroduodenal disease

6.1 Introduction

The structure and function of the *cag* pathogenicity island (*cag*PAI) and genes therein have been described in Chapter 4.4.2. The production of the CagA protein and presence of the majority of genes in the *cag* pathogenicity island (*cag*PAI) has been associated with clinically significant gastroduodenal disease in some studies but not in others [13,14,200,201,287,288]. A structural subtype of *cagA* may be associated with gastric carcinoma and atrophy [7,202,218], while an intact, complete, *cag* PAI is highly associated with duodenal ulceration in *H. pylori* isolates from France and Japan [216,221]. Patients with disease may, however, harbor strains with partial or complete deletions of the *cag* PAI [216,221]. Little, however, is known about the structure of the island in patients with gastric adenocarcinoma.

The structure of the *cag*PAI in South African isolates has not previously been studied. In this study we hypothesized that *H. pylori* isolates from different clinical diseases would exhibit variability in their *cag*PAI and that there would be an association between the type of *cag*PAI and the type of *vacA* allele. The presence and structural organization within key regions of the *cag*PAI were therefore investigated. The regions targeted and tested by means of specific polymerase chain reaction (PCR) assays were the functionally important *cagA*, *cagE*, and *cagM* genes in the *cagI* region, and two *cagII* marker genes, *cagT* and *cag6-7*. Strains which were "PCR-negative" for these *cagII* marker genes were analyzed for *cag5-10* (which encompasses the *virD4* homologue) as well as the downstream *cag13* gene. In addition, the integrity of the *cag*PAI was tested by analyzing the presence of *cagQ~S/T* amplicons. Finally, *IS605* (*tnpA/B*) was tested for as a potential insertion between *cagI* and *cagII*, and the prevalence of *IS606* was determined.

6.2 Materials and Methods

6.2.1 H. pylori strains and DNA

DNA prepared from 109 *H. pylori* isolates from 86 infected patients (Cf. section 5.2.1.) was used to analyze the presence of the *cagA* gene, heterogeneity in the 3'-region of this gene,

and for analysis of the *cag* PAI. Reference strains 26695 [41] and J99 [42] were used in this study.

6.2.2 PCR amplification and detection of amplified DNA products

Oligonucleotide primers used for PCR are listed in Table 6.1. Two sets of primers were used for each gene examined. PCR amplification was performed as previously described (cf. Chapter 5.2.3) [283,284]. Following initial denaturation, 94 °C for 1 min, each reaction consisted of 35 cycles of denaturation at 94 °C for 1 min., annealing and extension for 2-3 min. and final extension at 72 °C for 10 min. Annealing temperatures were set at 50 °C for cag2/cag4, picBF/R, 53 °C for cag7/12, cag8/9, cag10/11, cag13/14, cag9/10, cag7/10, cag11/12 and 55 °C for F1, B1, FB2F, RB3R, cagMF, cagMR, cag6-7F, cag 6-7R [10,193,216,220,221]. Twenty microliters of each PCR mixture was subjected to gel electrophoresis on 1% agarose gels and a 100 bp DNA ladder (Roche Diagnostics, South Africa) was used as a size marker. Long-distance PCR was performed with the Expand™ PCR System (Roche Diagnostics) as recommended by the supplier [289]. Briefly, samples were incubated in Tris-reaction buffer including $MgCl_2$ [22.5mM], detergents (Tween-20 and Nonidet P40 [0.5% v/v]), nucleotides (500uM) and the enzyme Pwo/Taq mix, and subjected to PCR. Following initial denaturation, 92 °C for 2 min, each reaction consisted of 35 cycles of denaturation at 92 °C for 30 s, annealing for 1 min., and extension for 24 min. and final extension at 68 °C for 10 min. Annealing temperatures were as for standard PCR. Twenty microliters of each PCR mixture was subjected to gel electrophoresis on 0.8% agarose gels and a 564-23,130 bp DNA ladder (Roche Diagnostics, South Africa) was used as a size marker.

Table 6.1 Primers used to identify *cag*PAI genes.

Gene	HP No.#	*Primer*	Primer Sequence	Location */+	Size (bp)	Ref
cagA 5'	HP547	F1 B1	5'-GATAACAGGCAAGCTTTTGAGGGA 5'-CTGCAAAAGATTGTTTGGCAGA	19119 19468	349	202
cagA 3'	HP547	cag2 cag4	5'-GGAACCCTAGTCGGTAATG 5'-ATCTTTGAGCTTGTCTATCG	21498 21947	449	193
cagE	HP544	picBF picBR	5'-TGTTTGGTTTCCCTG 5'-ACGCATTCCTTAACG	16998 15663	1335	10
cagE	HP544	cagEF cagER	5'-TCTATAAAGAGAGAGGTGTT 5'-GGCTAATCTTTGGTAATCAG	17170 14451	2719	221
cagM	HP537	cagMF cagMR	5'-ATGCTTGCAAAAATTGTTTT 5'-CTATTCAAAGGGATTATTCT	7772 8902	1130	221
cagM	HP537	cag5 cag6	5'-ACAAATACAAAAAAGAAAAAGAGGC 5'-ATTTTTCAACAAGTTAGAAAAAGCC	8022 8608	586	216
cagQ~cagS	HP535 HP534	CAG12 CAG7	5'-GCAATCATTGAGAAGAGTTTTCGC 5'-TTTGGTTGGTAATGGTTTTGGTAGC	1945 6268		216
cagQ~cagT	HP535 HP532	cagTF2 cagQR	5'-GTTTGCTCAGTGGTAAGTGA 5'-ATGCTTCCTACTAAAACACG	1167 6569		221
cagT	HP532	CAG13 CAG14	5'-TCTAAAAAGATTACGCTCATAGGCG 5'-CTTTGGCTTGCATGTTCAAGTTGCC	610 1100	590	216
cagT	HP532	cagTF cagTR	5'-ATGAAAGTGAGAGCAAGTGT 5'-TCACTTACCACTGAGCAAAC	21480 22322	842	221
cag13	HP527	cag13F cag13R	5'-GCTAGAGAAAAGGCTGTTGC 5'-TGGCGTTAATAGTGGCAATA	12771 12369	402	221
cag6-cag7	HP520 HP521	cag67F cag67R	5'-ATGGCAACATGGAGATGGTT 5'-TTAGTTTCCTTTTTTTTCAG	4289 5174	885	221
cag5-cag10	HP519 HP524	LECF cag10R	5'-ACATTTTGGCTAAATAAACGCTG 5'-TGGGTTCAAGCGAACTGTGA	3920 7290	3370	221
tnpA		CAG10 CAG11	5'-ATC AGT CCA AAA AGT TTT TTC TTT CC 5'-TAA GGG GGT ATA TTT CAA CCA ACC G	4270 4614	344	216
tnpB		CAG8 CAG9	5'-CGC TCT CCC TAA ATT CAA AGA GGG C 5'-AGC TAG GGA AAA ATC TGT CTA TGC C	5079 5648	569	216
IS606		FB2-F RB3-R	5'-GGA GGG TAG TTG ATA AGC AAA TC 5'-GTT TAG ACT TTA ACA CCC TAC GG		1070	220

= HP numbers from *H. pylori* strain 26695 (GenBank Accession No. AE000511)
*/+ Location = position on *H. pylori* (GenBank Accession No. U60176 or AC000108)

6.2.3 Sequencing of the 3'-end of the cagA gene

The 3'-region of *cagA* was amplified using primers cag2/cag4 in twelve South African isolates. PCR products were gel extracted (QIAEX II gel extraction kit, Qiagen, Cape Town, South Africa) and sequenced on an ABI PRISM 377 automated sequencer (ABI, Foster City, California, USA) using the ABI PRISM ™ BigDye ™ terminator cycle sequencing reagent kit with AmpliTaq ® DNA polymerase FS (PE Biosystems, Johannesburg, South Africa) as described previously [290]. PCR and direct sequencing were performed at least twice to determine DNA sequences for each strain.

6.2.4 Computer analysis

DNA and protein sequences were analyzed using the National Center for Biotechnology Information (NCBI) server (USA) and META Predict Protein (which includes the JPRED, NetPhos, TopPred and DAS servers [Columbia University, USA]) [291-294]. JPRED is a consensus method for protein and secondary structure prediction, NetPhos is a neural network-based method for predicting potential phosphorylation sites at serine, threonine and tyrosine residues, TopPred predicts the location and orientation of transmembrane sequences using hydrophobicity patterns by applying the "positive-inside" rule and DAS predicts the location of transmembrane using hydrophobicity patterns. Internet-based searches were preformed at NCBI, The Institute for Genome Research (TIGR, Maryland, USA) and Astra-Zeneca (Boston, USA).

6.2.5 Statistical methods

Data from non-identical strains (n = 109) were analyzed using the chi-squared test or Fisher's exact test as appropriate. For multiple regression analysis, the typing data were summarized into two-way tables. Each table had 109 rows and a column for the *vacA* genotype, *cag*PAI status and pathology. The presence or absence of each character was binarily coded, present = 1, absent = 0. All analyses were performed with STATISTICA © software (Gaithersburg, Maryland, USA). Probability levels of < 0.05 were considered statistically significant.

6.3 Results

6.3.1 Prevalence of the gene cagA in 109 H. pylori isolates

One hundred and six (97%) of 109 strains and the two reference strains were *cagA*$^+$ utilizing primers F1/B1 to identify a 349 bp conserved amplicon from the 5'-region of the gene. The gene *cagA* was present in 35 (97%) of 36 strains isolated from patients with peptic ulceration and 45 (96%) of 47 strains from patients with gastritis alone. All 26 isolates from patients with gastric adenocarcinoma were *cagA*$^+$. One hundred and six (97%) of 109 strains had identifiable PCR products with primer set cag2/cag4. All three of the strains which did not have an amplifiable conserved 5'-*cagA* region, had identifiable amplicons with this second primer set. This demonstrates that *cagA* was present in all isolates.

6.3.2 Analysis of sequence variation in the 3'-region of the gene cagA.

PCR fragments of differing length (approximately 450-750 bp) from twelve different South African isolates were sequenced, the amino acid composition determined, and compared to HP547 from strain 26695 and JHP495 from strain J99 (Figure 6.1). The conformation and

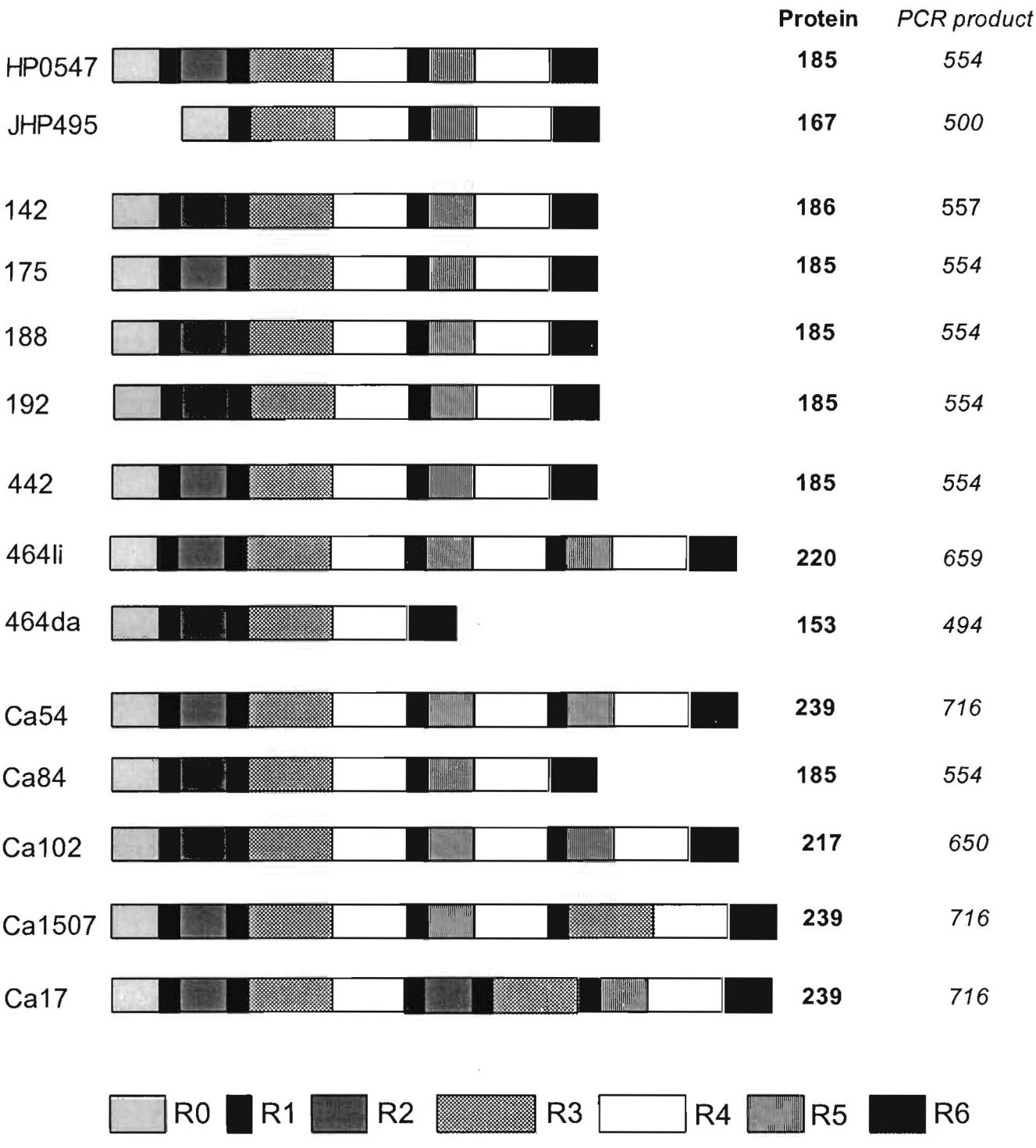
number of repeats in this part of the protein were analyzed using previously described methods [218,295] and is diagrammatically illustrated in Figure 6.2.

Figure 6.1 Deduced amino acid sequences of an internal segment of the variable (3'-region) of *cagA*.

```
HP0547:     NEK*FKNFNNNNNN**GLKNS*****TEPIYA KVNKKKTGQVASPE EPIYT
JHP495:     NAKL*GNFNNNNNNN*GLENS*****TEPIYT ********************** *****
142:        NEK*FKNFNNNNNNN*GLKNG*****GEPIYA KVNKKKTGQAASPE EPIYT
175:        NEK*FKNFNNNNNNN*GLKNG*****GEPIYA QVNKKKTGQVASPE EPIYA
188:        NEK*FKNFNNNNNNN*GLKN*******EPIYA KVNKKKTGQVASPE EPIYT
192:        NEK*FKNFNNNNNNN*GLKNGKDKGPEEPIYA QVNKKKTGQVASPE EPIYV
442:        NEK*FKNFNNNNNN**GLKNS*****TEPIYA KVNKKKTGQAASLE EPIYA
464da:      EAKL*GNFNNNNNNNNGLKNS*****TEPITA KVNKNKTGQVASPE EPITT
464li:      EAKL*GNFNNNNNNNNGLKNS*****TEPITA KVNKNKTGQVASPE EPITT
Ca54:       NEK*FKNFNNNNNNN*GLKNGKDKGPEEPIYA KVNKKKAGQAASPE EPIYA
Ca84:       NAKL*GNFNNNNNNN*GLKN*******EPIYA KINKKKAGQAASLE EPIYT
Ca102:      NAKL*GNFNNNNNNN*GLKN*******EPIYA KVNKKKTGQAASLE EPIYA
Ca1507:     NEK*FKNFNNNNNN**GLKN*******EPIYA QVNKKKTGQVASPE EPIYT
Ca17:       NEK*FKNFNNNNNN**GLKNS*****TEPIYA KVNKKKTGQVASPE EPIYT
                          R0              R1          R2          R1

HP0547:     QVAKKVNAKIDRLNQIA*SGLGGVGQAAGFP LKRHDKVDDLSKVGLSASP*EPIYA
JHP495:     QVAKKVKAKIDRLDQIA*SGLGDVGQAASFL LKRHDKVDDLSKVGLSANH*EPIYA
142:        QVAKKVNAKIDRLNQIA*SGLGGVGQAAGFP LKKHDKVDDLSKVGRSVSP*EPIYA
175:        QVAKKVTQKIDQLNQAA*SGFGGVGQA*GFP LKRHDKVEDLSKVGRSVSP*EPIYA
188:        QVAKKVNAKIDRLNQAA*SGLGGVGQAAGFP LKRHDKVDDLSKVGLSASH*EPIYA
192:        QVAKKVTQKIDQLNQAVTSGFGGVGQA*GFP LKRHDKVEDLSKVGRSVSPEEPIYA
442:        QVAKKVNAKIDRLNQIA*SGLGGVGQAAGFP LKKHDKVDDLSKVGRSVSP*EPIYA
464da:      QVAKKVNAKIDRLNQVA*SGLGGVGQAAG** ************************
464li:      QVAKKVNAKIDRLNQVA*SGLGGVGQAAGFP LKRHDKVDDFSKVGLSASP*EPIYA
Ca54:       QVAKKVNAKIDRLNQIA*SGLGGVGQAAGFP LKRHDKVEDLSKVGRSVSP*EPIYA
Ca84:       QVAKKVNAKIDRLNQAA*SGLGGVGQAAGFP LKKHDKVDDLSKVGRSVAL*EPIYA
Ca102:      QVAKKVNAKIDRLNQIA*SGLGGVGQAAGFP LKRHDKVDDLSKVGRRVSP*EPIYA
Ca1507:     QVAKKVTKKIDQLNQAA*SGLGGVGQAAGFP LKRHDKVDDLSKVGRSVSP*EPIYA
Ca17  :     QVAKKVNAKIDRLNQIA*SGLGGVGQSSAFP LKRHDKVDDLSKVGLSASP*EPIYA
                          R3                          R4          R1

HP0547:     ************************************************************
JHP495:     ************************************************************
142:        ************************************************************
175:        ************************************************************
188:        ************************************************************
192:        ************************************************************
442:        ************************************************************
464da:      ************************************************************
464li:      ************************************************************
Ca54:       ************************************************************
Ca84:       ************************************************************
Ca102:      ************************************************************
Ca1507:     ************************************************************
Ca17:       KVNKKKTGHVASPE EPIYA QVAKKVNAKIDRLNQIASGLGGVGNAGFPP EPIYA
                  R2          R1                  R3                  R1
```

```
HP0547:     TIDDLGGPFP L*KRHDKVDDLSKVGRS**********************************
JHP495:     TIDDLGGPFP L*KRHDKVDDLSKVGLS**********************************
142:        TIDDLGGPFP L*KRHDKVDDLSKVGLS**********************************
175:        TIDDLGGSFP L*RRSAKVEDLSKVGLS**********************************
188:        TIDDLGGPFP L*KRHDKVDDLSKVGLS**********************************
192:        TIDDLGGSFP L*RRSAKVEDLSKVGLS**********************************
442:        TIDDLGGPFP L*KRHDKVDDLSKVGLS**********************************
464da:      ********FP L*KRHDKVDDLSKVGLS**********************************
464li:      TIDDLGGPFP L*KRHDKVDDLSKVGLS**********************************
Ca54:       TIDDLGGPFP LLKRHDKVDDLSKVGRS**********************************
Ca84:       TIDDLGGPFP L*KRHDKVDNLSKVGLS**********************************
Ca102:      TIDDLGGPFP L*KRHDKVDDLSKVGRS**********************************
Ca1507:     TIDDLGGPFP L*KKHDKVEDLSKVGLSASPEEPIYT QVAKKVTQKIDRNLNQIASGLGGVG
Ca17:       TIDDLVGPFP L*KRHDKVDDLSKVGLS**********************************
                 R5          R4                R1          R3

HP0547:     ************************************************************
JHP495:     ************************************************************
142:        ************************************************************
175:        ************************************************************
188:        ************************************************************
192:        ************************************************************
442:        ************************************************************
464da:      ************************************************************
464li:      **********************VSPEPIYA TIDDLGGPFP LKRHDKVDDLSKVGR
Ca54:       **********************VSPEPIYA TIDDLGGPFP LKRHDKVDDLSKVGR
Ca84:       ************************************************************
Ca102:      **********************VSPEPIYA TIDDLGGPFP LNRHDNVDDLSKVGL
Ca1507:     QAAGFP LKKHDKVEDLSKVGL**********************************
Ca17:       ************************************************************
                       R4                R1        R5          R4

HP0547:     *RNQELAQKIDNLN*QAVSEAKAGFFGNL
JHP495:     *REQKLTQKIDNLN*QAVSEAKASHFDNL
142:        *RNQELAQKIDNLS*QAVSEAKAGFFANL
175:        *RERELTQKIDSLS*QAVSEAKAGFFGNL
188:        *RNQELAQKIDNLS*QAVSEAKAGHFANL
192:        *RGQELTQKIDSLS*QAVSEAKAGFFANL
442:        *RERELAQKIDNLN*QAVSEAKAGFFANL
464da:      *RNQKLAQKIDNLN*QAVSEAKAGFFANL
464li:      *RNQKLAQEIDNLN*QAVSEAKAGFFGNL
Ca54:       SRDQELAQKIDNLS*QAVSEAKAGFF*NL
Ca84:       *REQQLKQKIDNLN*QAVSEAKAGFFANL
Ca102:      *RN*QELAQKIDSLSQAVSEAKAGFFRNL
Ca1507:     SRNQNLAQKIDNLN*QAVSEAKAGFFGNL
Ca17:       *RNRELAQKIDNLN*QAVSEAKAGFFGNL
                           R6
```

Figure 6.2 Diagrammatic representation of the variability in the 3'-region with predicted protein size and PCR products (bp).



The multi-asparagine region (R_0) ranged in size between 5 and 7 Asn repeats. This was followed by an EPIY(A/T), R_2 and a second EPIY(A/T/V) which is similar to HP547 in strain 26695 but not to JHP495 where the R_2 and the second EPIYA are deleted. All isolates exhibited an R_3 region, an R_4 region and a third EPIYA except for isolate 464da in which the latter two were deleted. Alteration in the latter half of the gene including additional R_2/EPIYA repeats and EPIYA/R_3/R_4 or EPIYA/R_5/R_4 were present in five (42%) of the 12 South African isolates. These alterations and truncations resulted in predicted proteins varying in size from 153 to 239 amino acids with predicted PCR fragment lengths ranging from 494 to 716 bp. DNA analysis demonstrated significantly more homology with *cagA* from the European strains (HP0547 and JHP495; 73-94% identity, 75-95% positivity) than with *cagA* from four Japanese strains JK25 (GenBank accession number AF043487; 52-57% identity), JK253 (GenBank accession number AF043489; 33-44%), JK269 (GenBank Accession No. AAC02084; 38-44%) and JK271 (GenBank accession number AF043488; 38-43%). These results confirm that South African *cagA* in this study is "non-Asian" in origin and strongly suggests that these *cag*$^+$ strains will potentially share more sequence identity with European strains [216,222] than with Japanese strains [221].

Analysis of the putative protein secondary structures and topology and potential phosphorylation sites are given in Table 6.2.

Table 6.2 Protein characteristics of *cagA* 3'-variants

		JHP495	*HP547*	*142*	*175*	*188*	*192*	*Ca54*	*Ca84*	*Ca102*	*Ca1507*	*Ca17*
Amino acids (n)		167	185	186	185	185	185	239	185	217	239	239
#Proline (%)		3.0	5.4	5.3	4.7	4.7	5.1	6.9	4.0	6.1	5.5	6.4
^{3}Phospho-S (n)		4	6	5	9	4	9	9	4	9	8	7
^{3}Phospho-T (n)		2	1	1	2	1	2	2	1	2	1	1
^{3}Phospho-Y (n)		2	3	3	3	3	3	4	3	4	4	5
TP	**PTM (n) +Position (score)*	None	One 54-74 (0.606)	One 56-76 (0.642)	None	None	One 60-80 (0.707)	One 53-73 (0.606)	None	Two 52-72 (0.614) 142-162 (0.684)	None	None
D	*PTM (n) Position:*	None	One 60-66	One 61-67	None	One 62-65	None	One 62-63	None	None	None	None
#Helix (%)		42.0	40.3	30.7	36.2	34.5	34.6	28.0	44.6	26.5	38.1	35.5
#Sheet (%)		0.8	3.4	8.7	4.7	5.4	10.9	4.8	1.4	12.2	1.0	1.0
#Other (%)		57.2	56.4	60.7	59.1	60.1	54.5	67.2	54.0	61.3	60.9	63.5

TP = TopPred, D = DAS, # = identified using JPRED, ^{3} = identified using NetPhos

*PTM = putative transmembrane segment, +Position = amino acid position

Cysteines were absent in all samples while the percentage of proline ranged from 3.0-~7.0%. This was reflected in the differences in the percentage helix/sheets formed, and in the number of potential immunogenic surface peaks. In general, the longer the 3'-length, the greater the number of transmembrane segments and surface exposure. This correlated with the percentage of proline residues ($p < 0.02$). In addition, longer variants (which were invariably from gastric cancer patients) exhibited significant differences in the potential number of phosphorylatable tyrosine residues ($p < 0.02$) but not serine or threonine residues compared to short variants. Similar differences in topology, and in the potential number of phosphorylatable tyrosine residues but not serine or threonine residues were noted in the Japanese strain JK269 (Strain C) which has been positively associated with gastric cancer [218].

6.3.3 Analysis of size variation in the gene cagA.

The results of size variation in the 106 *cagA3'*$^{+}$ isolates are shown in Table 6.3. Based on the sequence analysis (cf. Chapter 6.3.2) isolates could be divided into groups of differing fragment lengths of < 600 bp, 600-700 bp and > 700 bp. Amplicons smaller than 600 bp were found more frequently in *H. pylori* strains isolated from patients with gastritis alone (43 of 45 [96%]) than from patients with peptic ulceration (27 of 35 [77%], $p < 0.02$) and in those from patients with gastric adenocarcinoma (16 of 26 [61%]; $p < 0.003$) (Table 6.3).

Table 6.3 Relationship between *cagA*-3' and disease

No. of Strains	Fragment length of *cagA* 3'-amplicon		
	< 600 bp	600 – 700 bp	> 700 bp
Gastritis alone (n = 45)*	43 (96%)[a,b]	2 (4%)	0 (0%)
Peptic ulceration (n = 35)**	27 (77%)	5 (14%)	3 (8%)
Adenocarcinoma (n = 26)	16 (61%)	2 (8%)	8 (31%)[c,d]
p-value	[a]< 0.02 [b]< 0.003	p = NS	[c]< 0.0002 [d]< 0.04

*Two gastritis and **one peptic ulcer isolate did not have identifiable 3'-amplicons

[a,d]Compared with peptic ulceration, [b]Compared with adenocarcinoma, [c]Compared with gastritis.

Amplicons of 600-700 bp were not differentially distributed between the groups. PCR fragments of > 700 bp were found more often in patients with gastric adenocarcinoma (8 of 26 [31%]) than in patients with gastritis alone (0 of 45 [0%], $p < 0.0002$) and from patients with peptic ulceration (3 of 35 [8%]; $p = 0.037$).

6.3.4 Predictive value of typeI strains

Type 1 (*vacAs1*$^+$*cagA*$^+$) was found in all peptic ulcer disease (36 of 36) strains ($p < 0.00001$ vs. gastritis alone) and in all gastric adenocarcinoma (26 of 26) strains ($p < 0.00002$ vs. gastritis alone) compared to 57% (27 of 47) of strains from patients without clinically significant disease (gastritis alone). The sensitivity of type I strains as a marker for gastric disease (either GCa or PUD) is 100% based on the data generated in this study (Table 6.4), while the specificity of type I strains as a marker is 43%. The predictive values for type I strains ranges between 49-57%.

Table 6.4 Predictive value of type 1 strains (*vacAs1*$^+$*cagA*$^+$) for clinically significant disease

Diagnosis	PPV	NPV	SE	SP
PUD	0.57	1.0	1.0	0.43
GCa	0.49	1.0	1.0	0.43

PUD = peptic ulcer disease, GCa = gastric adenocarcinoma

PPV = positive predictive value, NPV = negative predictive value, SE = sensitivity, SP = specificity.

6.3.5 PCR analysis of cagI.

H. pylori isolates were analyzed with two different primer sets for the *cagA* (HP547), *cagE* (HP544) and *cagM* (HP537) genes of the *cagI* region. The agreement between primer sets for *cagA* was 97%, *cagE* (100%) and *cagM* (98%). Isolates were considered positive if one primer set gave a successful PCR amplification. The reference strains 26695 and J99 were positive for each primer combination used. Overall, eighty-eight (81%) of 109 strains had all the *cagI* marker genes. All one hundred and nine of the strains were $cagA^+$ utilizing primers F1/B1 or cag2/4 (Figure 6.3).

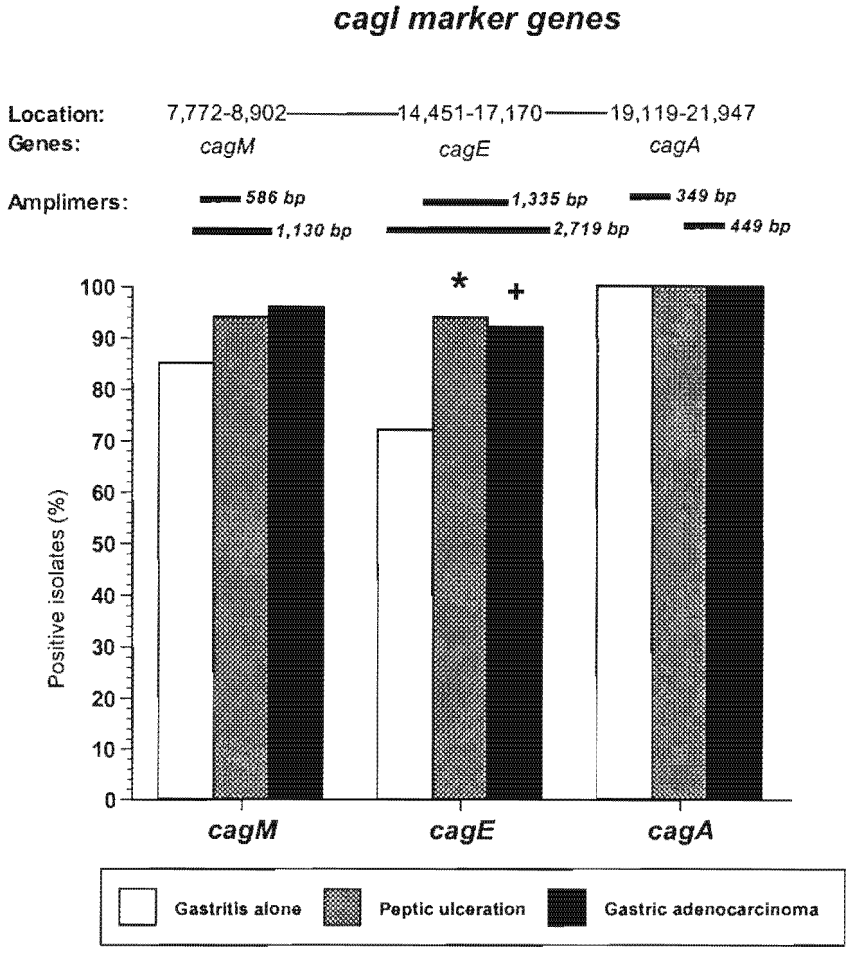


Figure 6.3 Schematic of *cagI* with the PCR amplicons of the three genes analyzed (top). Genes and positions are from GenBank Accession No. U60176. Prevalence of target genes in the different disease groups (bottom). *$p < 0.009$, $^+p < 0.05$ (each vs. gastritis alone).

The gene *cagE* was present in significantly more strains isolated from patients with peptic ulceration (34 of 36 [94%]; $p < 0.009$) and strains from patients with gastric adenocarcinoma (24 of 26 [92%]; $p < 0.04$) than strains isolated from patients with gastritis alone (34 of 47

[72%]). The gene *cagM* was present in 85% of isolates from patients with gastritis alone, 94% PUD isolates and 96% of gastric adenocarcinoma isolates (p = NS). Reference strains 26695 and J99 had all the markers for *cagI*. These results demonstrate that functionally important elements of the *cagI* region (*cagA-cagM*) were present in the majority of strains irrespective of disease status. However, *cagE*, which has previously been determined to play a critical role in CagA translocation and IL-8 induction [212], was not detected by PCR utilizing two different primer sets in a quarter of isolates from patients with no clinically significant disease.

6.3.6 PCR analysis of cagII.

H. pylori isolates were analyzed with two different primer sets for *cagT* (HP532) which is present at the 3'-end, and with one primer set for *cag6-7* (HP520-521) present at the 5'-end of this region. The agreement between primer sets for *cagT* was 87%. Overall, sixty-six (61%) of 109 strains were both *cagT*$^{+}$ and *cag6-7*$^{+}$. Thirty-five (97%) of 36 isolates from patients with peptic ulceration were *cagT* $^{+}$, compared to 24 (92%) of 26 isolates from patients with gastric adenocarcinoma and 30 (64%) of 47 isolates from patients with gastritis alone ($p < 0.0002$ vs. PUD, $p < 0.007$ vs. GCa) (Figure 6.4). Significantly more isolates from peptic ulcer patients (29 of 36 [81%]) and gastric adenocarcinoma patients (19 of 26 [73%]) were *cag6-7*$^{+}$, compared to isolates from patients with gastritis alone (21 of 47 [45%], $p < 0.0009$ vs. PUD, $p < 0.02$ vs. GCa). Both reference strains produced the expected size *cagT* and *cag6-7* amplicons.

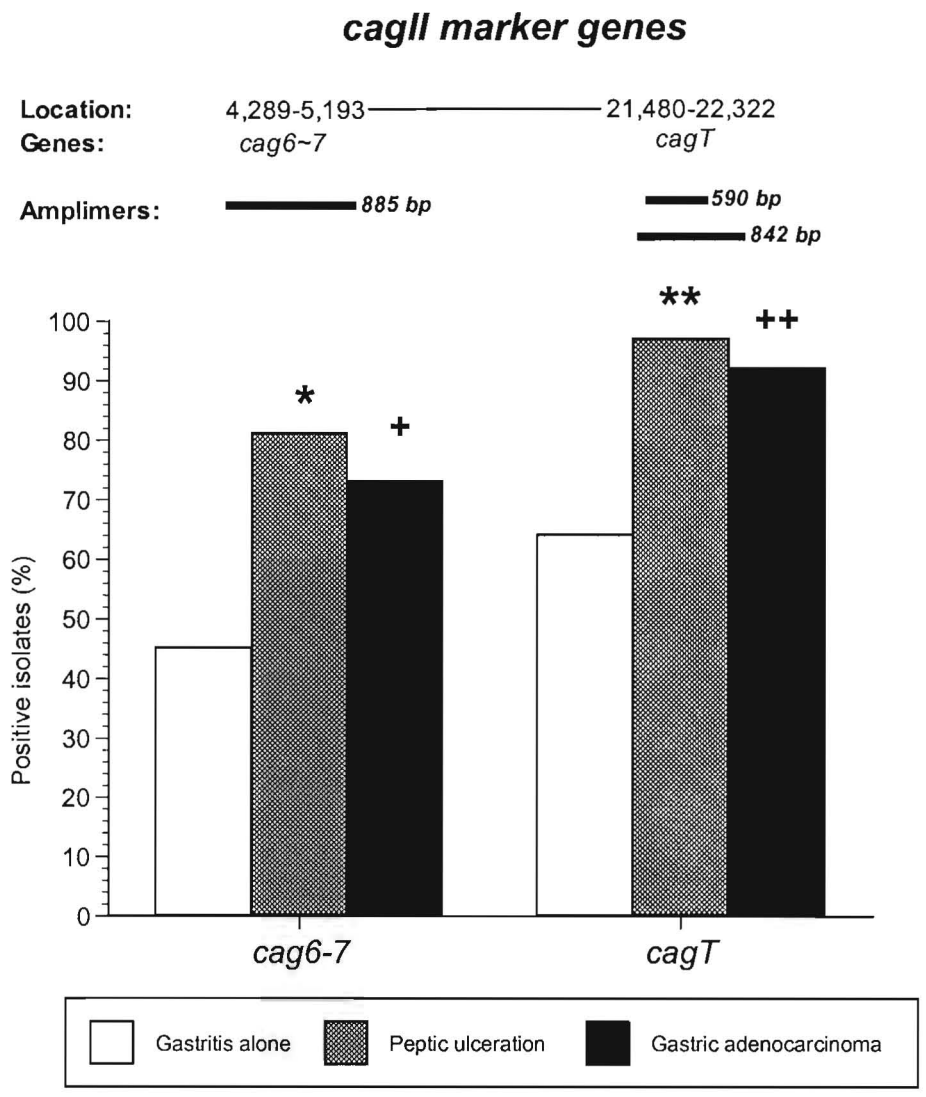


Figure 6.4 Schematic of *cagII* with the PCR amplicons of the three genes analyzed (top). Genes and positions are from GenBank Accession No. AC000108. Prevalence of target genes in the different disease groups (bottom). *$p < 0.0002$, **$p < 0.0009$, $^{+}p < 0.007$, $^{++}p < 0.05$ (all vs. gastritis alone).

In order to exclude sequence heterogeneity at primer annealing sites in the 5'-region of the *cag*PAI as a reason for negative PCR results, forty isolates which were PCR-negative for *cag6-7* were also examined for *cag5-10* (HP519-524). This region encompasses the *vir*D4 homologue, a putative toxin, and should result in a PCR product of 3,370 bp (predicted from GenBank Accession No. AC000108). Twenty-six isolates (65%) of the 40 isolates had PCR amplicons ranging in size from 880-2,902 bp compared to the expected 3,370 bp in all ten *cag6-7*$^{+}$ isolates tested. We next tested whether an additional *cagII* marker gene, *cag13*

(HP527), which is between *cag10* and *cagT* was present in these twenty-six isolates. Twenty-five (96%) of these isolates were also *cag13*$^{+}$ (as were all ten *cag6-7*$^{+}$ isolates tested). Long PCR using primers to examine the entire *cagII* region (*cag5-cagT*) was performed on the one isolate negative for *cag13*. This should result in a PCR product of 18,402 bp (predicted from GenBank Accession No. AC000108). A product of 8,396 bp was amplified. These results suggest that twenty-six isolates, which were all PCR-negative (using two different primer sets) for *cagT*, contain a partial *cagII* (Figure 6.5).

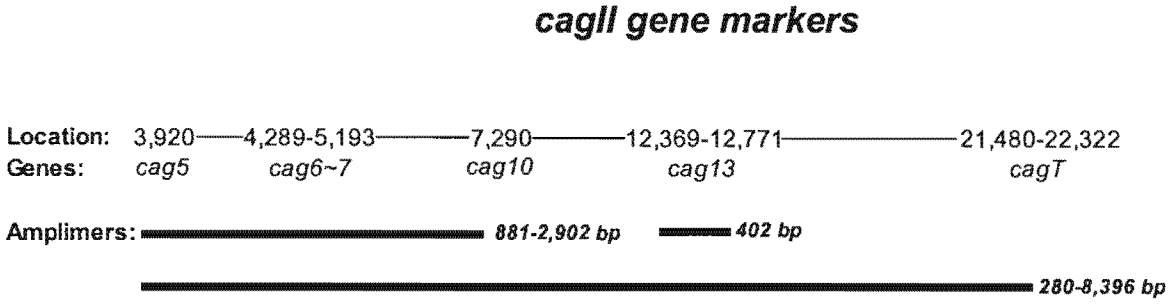


Figure 6.5 Variability of *cagII* in forty isolates which are PCR-negative for *cag6~7*. Genes and positions are from GenBank Accession No. AC000108. Twenty-five isolates had variable length PCR amplimers (881-2,902 bp) for *cag5-10* (3,370 bp predicted), and were PCR-positive for *cag13*. Eleven isolates (all *cag13*-negative) had variable length PCR amplimers (280-8,396bp) for *cag5-T* (18,402 bp predicted). Four isolates were *cagII* PCR-negative.

Thereafter we investigated the fourteen isolates which had lost the 5'-region of *cagII* (PCR-negative for both *cag6-7* and *cag5-10*) using long PCR to examine the entire *cagII* region (*cag5-cagT*). PCR amplicons of 280-3,105 bp (compared to a product of 18,402 bp predicted from GenBank Accession No. AC000108) were generated in ten of the 14 isolates. These results suggest that these isolates had lost the majority of the *cagII* region. Analysis of the remaining four *cag5-T* PCR-negative isolates demonstrated that one isolate had lost all the genes between *cag5* and *cagM*, one isolate, all the genes between *cag5* and *cagE* and two isolated contained only *cagA*.

These results demonstrate that a complete, intact *cagII* region (*cag6-cagT*) is present in significantly more isolates from patients with peptic ulcer disease (81%, $p = 0.0001$) than in patients with gastritis alone (38%). Analysis of the distribution of *cag*PAI gene products demonstrated a significant association with PUD ($X^2 = 25.35$, $P = 0.00012$). Seventy-three percent of isolates from patients with gastric adenocarcinoma had a complete, intact *cagII* (p

= 0.004 vs. gastritis alone) while downstream elements of the *cagII* region (*cag10-cagT*) were present in 100% of these isolates ($p < 0.005$ vs. gastritis alone). The distribution of *cag*PAI gene products was similarly, but not as strongly, associated with gastric adenocarcinoma ($X^2 = 10.14$, $P < 0.05$).

6.3.7 Relationship between gene markers and vacA status

As previously demonstrated (cf. Chapter 5), *vacA* s1 occurred significantly more often in isolates from patients with peptic ulceration ($p < 0.00001$) or gastric adenocarcinoma ($p < 0.0001$), while *vacA* s2 invariably occurred in patients with gastritis alone. In addition, the *vacA* midregion (m1 subtype) was present more often in patients with clinically significant disease ($p < 0.01$). Multiple regression analysis demonstrated a statistically significant relationship between *vacA* s1 alleles and disease ($p = 0.001$ [PUD], $p = 0.014$ [GCa]).

When analyzing the data by *vacA* status (Table 6.5), there was a significant difference in the distribution of alleles between the different *cag*PAI groups ($X^2 = 41.5$, $p < 0.00004$). A strong association was noted between *vacA* s1m1 and strains containing the entire genetic information of *cag*PAI. Fifty-four (77%) of 70 *vacA* s1m1 strains had all the genetic markers (*cag6-cagA*) compared to 10 of 19 (53%, $p < 0.04$) *vacA* s1m2 and 2 of 17 (12%, $p < 0.00001$) *vacA* s2m2 strains. Singificantly more strains with *vacA* s1m2 (37%, $p < 0.008$) and *vacA* s2m2 strains (71%, $p < 0.000001$) had intervening regions of the *cag*PAI which were not detected by PCR compared to strains with *vacA* s1m1.

Table 6.5 Relationship between *cag*PAI gene markers and *vacA* alleles

***cag* genes detected**	**HP numbers**	**s1m1 (n = 70)**	**s1m2 (n = 19)**	**s2m1 (n = 3)**	**s2m2 (n = 17)**
All tested genes detected	HP520-HP547$^+$	52 (77%)*#	10 (53%)	2 (67%)	2 (12%)
Some *cag* genes detected	HP524-HP547$^+$	6 (9%)	1 (5%)	1 (33%)	0 (0%)
	HP532-HP547$^+$	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	HP544-HP547$^+$	1 (1%)	1 (5%)	0 (0%)	2 (12%)
	Intervening sequences missing	*8 (11%)*#&	*7 (37%)*	*0 (0%)*	*12 (71%)*
Only *cagA* detected	HP547$^+$	1 (1%)	0 (0%)	0 (0%)	1 (6%)

*$p < 0.00001$ vs. *vacA* s2m2; #$p < 0.04$ vs. *vacA* s1m2; &$p < 0.008$ vs. *vacA* s1m2

6.3.8 Prevalence of cagQ-S/T amplification products

The primer combinations of *cag7/12* and *cagTF/QR* test both for the presence of the *cagQ~S/T* genes and also for joined *cagI* and *cagII* regions. The agreement between primer sets for *cagQ~S/T* was 78%. This is lower than for *cagI* but reflects the fact that these primer combinations identify different5'-genes. PCR amplification using these primer sets resulted in the correct amplicon sizes in both 26695 and J99. Overall, seventy-one (65%) of 109 strains were both *cagQ~S*$^+$ and *cagQ~T*$^+$. Eight-six percent of isolates from patients with peptic ulcer disease were positive for the 532 bp product predicted for an intact *cagQ~S* region, compared with only 20 (43%) isolates from patients with gastritis alone ($p < 0.0001$). Seventy-seven percent of strains (20 of 26) from patients with gastric adenocarcinoma were *cagQ~S*$^+$ ($p < 0.007$ vs. gastritis). The 842 bp product predicted for an intact *cagQ~T* was present in eighty-nine percent of isolates from patients with peptic ulcer disease compared with only 24 (51%) isolates from patients with gastritis alone ($p < 0.0001$). All strains from patients with gastric adenocarcinoma were *cagQ~T*$^+$ ($p < 0.00001$ vs. gastritis).

Twenty-eight isolates did not have an amplifiable *cagQ~S/T* gene product. One (3%) of the isolates was also *cagT*, *cag13* and *cag6-7* PCR-positive. This suggests that this isolate (from a patient with PUD) had a complete but spatially separated pathogenicity island. These results suggest that in the majority (89%, 26 of 28) of isolates where *cagQ~S/T* were not detectable other genes in *cagII* would also not be present. There is also a strong association between specific *vacA* alleles and an absence of the *cagQ~S/T* amplicon. Significantly more *vacA* s2 isolates (13 of 20 [65%]) were *cagQ~S/T* PCR-negative compared to 14 of 83 (17%, $p < 0.0001$) *vacA* s1 strains, suggesting that the *vacA* s2 allele may be a surrogate marker for the loss of this region.

6.3.9 Prevalence of the insertion sequences, IS605 and IS606

While 26695 had an intact *IS605* sequence (*tnpA+tnpB*) detected by PCR and J99 only exhibited *tnpA*, only three South African isolates had an intact *IS605* sequence. All three had all had the marker genes for the *cag*PAI and yielded gene products with *cag7/12* and *cagTF/QR* primer sets suggesting that the complete insertion sequence was elsewhere in the genome. Analysis of the *cag*PAI deletion endpoints in the twenty-eight isolates which did not have an amplifiable *cagQ~S/T* gene product demonstrated that *IS605* (*tnpA*) was present in 6 (27%) of them suggesting that this insertion sequence may play a role in *cag*PAI disruption. Analysis of *IS605*, however, also demonstrated that *tnpA* was present in the majority of

isolates (95%), while *tnpB* was present in only 18 (24%) of isolates. This suggests that *tnpA* and *tnpB* occur most often as separate entities in the genome of South African samples.

In contrast to *IS605*, *IS606* was present in forty (54%) of the isolates ($p < 0.000001$ vs. *IS605*). There were no differences in distribution of this insertion sequence in either the disease groups (43% gastritis alone, 56% peptic ulceration, and 68% GCa, p = NS) or in isolates with (31 [60%]) or without (9 [41%]) a *cagQ~S* amplicon. These findings suggest that complete insertion sequences are not involved in alterations of the PAI in these South African isolates but that *tnpA* from *IS605* might be involved in a quarter of the isolates.

6.3.10 Predictive value of vacAs1$^+$cagPAI$^+$ strains

Strains (*vacAs1*) with all the information encoding *cagA* and the *cagPAI* were found in 29 of 36 (81%; $p < 0.00001$ vs. gastritis alone) peptic ulcer disease strains and in 18 of 26 (69%; $p < 0.0007$ vs. gastritis alone) gastric adenocarcinoma strains compared to 13 of 47 (28%) strains from patients without clinically significant disease (gastritis alone). The sensitivity of *vacA*s1/*cag*PAI as a marker for gastric disease (either GCa or PUD) is >80% (Table 6.6), while the specificity for this as a disease marker is 72%. The predictive values ranges between 58-69%.

Table 6.6 Predictive value of *vacAs1$^+$cagPAI$^+$* strains for clinically significant disease

Diagnosis	PPV	NPV	SE	SP
PUD	0.69	0.83	0.81	0.72
GCa	0.58	0.81	0.69	0.72

PUD = peptic ulcer disease, GCa = gastric adenocarcinoma

PPV = positive predictive value, NPV = negative predictive value, SE = sensitivity, SP = specificity.

6.4 Discussion

In the present study, the results indicate that using a subset of previously defined, functionally important marker genes, the *cag* PAI appears complete and contiguous (*cag6-cagA*) in 65% of South African *H. pylori* isolates. One third of isolates had non-amplifiable gene regions in the island. Marker genes from the entire *cagII* region (*cag6-cagT*) were absent in ~90% of these cases. The caveat of over-interpretation of PCR results is recognized, as this methodology can fail for a number of reasons including heterogeneity at

primer annealing sites. Such heterogeneity may be one reason to explain our findings although we took care to use two established, different primer sets per gene site (these primer combinations have previously been used to identify *cag* genes in European populations [216,222]) to confirm negative forms. It is more likely, however, that the target regions are deleted. This is suggested both by the pattern of changes and by the evidence that South African *H. pylori* cag^+ isolates share more sequence homology with European than Asian strains [167].

All (100%) of tested strains, irrespective of the organization of the PAI, were $cagA^+$. Data on the *cag*PAI from the rest of Africa are scarce, but CagA appears to be commonly expressed [296]. Our data suggest dissociation between the presence of *cagA* and of other genes in the *cag*PAI island and further that analysis of the PAI may be a prerequisite for the investigation of any relationship with gastroduodenal disease processes. The nucleotide sequence of the gene *cagA* contains internal duplications of a 102 base pair fragment, encoding a proline rich region in the 3'-region [7]. Differences in this region have been suggested to generate proteins with different sizes and immunogenicities [7,202,218]. Comparable differences in sequences were found in the majority of South African isolates in this study. This suggests that such genetic alterations may be a common phenomenon in *H. pylori*. We evaluated whether there was a pathological significance to the variability in fragment length of the 3'-region of *cagA* in a population with a high prevalence of isolates containing this gene. The length of this region was shorter in South African strains isolated from patients with gastritis alone compared to those with peptic ulceration. This is in contrast to a German study which found the obverse [193]. In addition, significantly fewer gastric cancer isolates had gene fragments of this length (< 600 bp). The *cagA*-3' fragment length (600-700 bp) was not differentially distributed. In contrast, significantly more isolates from patients with gastric cancer had the longest fragment lengths (> 700 bp) than those from either patients with gastritis alone or peptic ulceration. These results are similar to the preliminary results from a Japanese study which demonstrated that a structural subtype of *cagA* (strain C) encoding a protein with a higher molecular weight and detectable by its larger size, was associated with gastric carcinoma and atrophy [218]. These data thus support the view that *cagA* variants may provide additional markers to distinguish disease-associated strains of *H. pylori*.

While the majority of isolates (~80%) from patients with peptic ulceration had all the marker genes for a complete *cag*PAI, less than half (~40%) of the isolates from patients with

gastritis alone had this pathogenic fingerprint. In contrast, significantly more isolates from patients with gastritis alone (23%) had regions of *cagII* (*cag6-cagT*) which were not detected compared to only three (8%) peptic ulcer disease isolates. This region includes the putative virulence factor, *cag10* (HP524) or *virD4* [168,176,214]. Analysis of the distribution of the virulence associated *vacA* alleles demonstrated that subtype s1/m1 was strongly associated with a complete island, whilst subtype s2/m2 was associated with deletions in *cagII*. These findings suggest the potential importance of genes in the *cagII* region for the pathogenesis of peptic ulcer disease and are supported by the observation of a significantly negative relationship between the virulence associated *vacA* s1 allele and deletions in *cagII*.

The association between genes in the *cag*PAI other than *cagA* and gastric adenocarcinoma has not previously been reported. Our results demonstrate significant differences between isolates from patients with gastritis alone and those with this disease. Specifically, an intact, frequently contiguous PAI was found more often in patients with cancer than in patients without clinically significant disease. The finding of a conserved pathogenicity element (*cag*PAI) shared by isolates from patients with gastric adenocarcinoma and peptic ulcer disease is of interest given the inverse relationship between these two diseases. Such a generalized association suggests that an intact PAI, while implying "virulence", may not be a specific marker for either of these diseases which is entirely compatible with the current understanding of the multi-factorial nature of the pathogenesis of these diseases. An alternate explanation may be sought in the demonstration that alterations in the 3'-gene sequence of *cagA* differentially identifies isolates from patients with gastric cancer [218,297,298]. It is possible that while an intact PAI (type IV secretion system) is necessary to deliver CagA into epithelial cells, it is the structural product of this gene which determines the degree of amino acid phosphorylation and which intracellular pathway (secretory or proliferative) a cell undergoes [215].

The grouping of clinical isolates into two broad families, type I and type II has been evaluated in this study. The data suggest that type I strains are strongly associated with gastroduodenal disease in these South African populations. The finding that all strains were *cagA* indicates that the *vacA* status may be an adequate surrogate marker for type I strains in the study population. Revising this classification to include strains which encoded all the information for the cag PAI did not diminish the ability to discriminate disease associated strains, but indeed increased both the positive predictive value as well as the specificity of this method as a diagnostic test.

The insertion sequence, *IS605*, was present in 25% of *cag*PAI deletion endpoints which supports the postulate that this DNA element may generate rearrangements and deletions in a small proportion of South African strains so as to result in sub-populations of organisms with differences in virulence [176,214]. Other potential mechanisms which may result in *cag* gene deletions include the observation that elimination of one part of the *cag*PAI may readily result in the loss of virulence of other parts of the genome, the possibility that organisms with such deletions are more readily cultured or that the non-random distribution of sequences may promote such losses. Irrespective of the mode of genetic deletion, it may be advisable to classify strains that carry the *cagA* gene and have internal deletions in the *cag*PAI as functionally cag^- rather than cag^+.

6.5 Conclusion

The presence of the gene *cagA* in all isolates, irrespective of associated pathology, suggests limited use of this as a measure of virulence. However, variability noted in the fragment length of the 3'-region of the gene correlates with disease (particularly gastric cancer) in this population and may prove to be a useful virulence marker. It may also provide a tool to differentiate between peptic ulcer disease and gastric cancer.

The presence of a contiguous *cag* PAI in sixty percent of patients suggests that this is conserved in most South Africans as it is in European and Asian populations [216,221,222]. The importance of genes in this island (particularly in *cagII*) to the pathogenesis of gastroduodenal disease in South Africa is, however, suggested by the prevalence of deletions in the 5'-region in patients with gastritis alone. In addition, the strong relationship between the virulent *vacA* type s1/m1 and the entire PAI, would seem to support the importance of both these elements to disease pathogenesis, although it appears that the relationship with clinically significant disease may be stronger for *vacA* alleles than for an intact *cag*PAI.

These studies suggest that a number of the associations established between *H. pylori* virulence factors and disease in other parts of the world apply to the South African setting. Analyses of these associations suggests that the relationship between specific genes (*cagA, cagE*) in the *cag*PAI and disease appear to be similar world-wide. There are, however, some interesting differences. The prevalence of *cagA* is extremely high. The widespread prevalence of this gene may have important ramifications and requires further study.

Chapter 7.

Relationship between *iceA* genotypes, *vacA* alleles and clinically significant disease

7.1 Introduction

The structure and function of the *iceA* genes are described in Chapter 4.4.3. Recently, a polymorphism of the *iceA* gene which may be clinically relevant has been reported [185]. In some populations (USA, Netherlands) *iceA1* strains appear to be much more prevalent among patients with ulcers [185,223], and *iceA1* may be a marker for peptic ulcer disease, independent of the genes *cagA* and *vacA* [185]. It appears that a combination analysis of *iceA* allele and *vacA* allele may be more useful for identifying strains associated with patients with peptic ulcer disease than analysis of each allele alone [185]. In another study, utilizing patients mainly from the Orient (South East Asia), no association was present [201]. The authors of the latter study suggest that the distribution of *iceA1* may reflect population clustering rather than virulence *per se*.

In contrast to *iceA1*, *iceA2* has no homology to known proteins and its structure reveals patterns of repeated protein cassettes [225]. More recently, the genetic organization and sequence heterogeneity of *iceA2* has been studied [224], revealing 5 distinct *iceA2* subtypes. While *iceA2* strains are more prevalent among patients with gastritis alone and non-ulcer dyspepsia [185], a statistically significant relationship between *iceA2* subtypes and disease has not yet been defined.

South African *H. pylori* isolates are characterized by an almost universal presence of *cagA*, but have differences in the 3'-region of *cagA* and the *vacA* gene which correlate significantly with clinically significant disease (c.f. Chapters 5 and 6). Our hypothesis was that *H. pylori* isolates from South African patients may also exhibit variability in their *iceA* alleles and that this variability may be related both to the expression of clinically significant disease and to *vacA* allelic status.

7.2 Materials & Methods

7.2.1 H. pylori strains and DNA

A total of 109 *H. pylori* isolates (*vacA* and *cagA* determined [Chapters 5 and 6]) were examined. Reference strains 26695 (*iceA1*) isolated from a patient in the UK with gastritis [41]

and J99 (*iceA2*) isolated from a US patient with duodenal ulcer disease [42] were used as *iceA1* and *iceA2* positive and negative controls, respectively, in this study.

7.2.2 Amplification of iceA by PCR

PCR reactions were performed as described (cf. Chapter 5.2.3) but a final $MgCl_2$ concentration of 3.5mM was used for *iceA1*. For amplification of the *iceA1* allele, forward primer *IceA1F* (5'-CGTTGGGTAAGCGTTACAGAATTT) and reverse primer *IceA1R* (5'-TCATTGTATATCCTATCATTACAAG) yielded a fragment of 558 bp (Figure 7.1). For *iceA2*, primers *IceA2F* (5'-GTTGTCGTTGTTTTAATGAA) and *IceA2R* (5'-GTCTTAAACCCCACGATTAAA) yielded a fragment of 120 bp. PCR was performed in a Sprint PCR (Hybaid, South Africa) under the following conditions: 4-minute preincubation at 94°C, followed by 30 cycles of 1 min. at 94°C, 1 min. at 62°C (*iceA1*) or 53°C (*iceA2*) and 1 min. at 72°C. Final extension was performed for 10 min. at 72°C.

Additional genotype-specific PCR assays that used primers for *iceA1* [*IceA1F5* (5'-GTGTTTTTAACCAAAGTATC) and *IceA1R4* (5'-CTATAGCCASTYTCTTTGCA)] and primers flanking the *iceA2* internal cassette were performed to confirm the presence of mixed *iceA1*$^+$/*iceA2*$^+$ isolates and to identify the size of the *iceA2* allele [185]. Primers *IceA2F6* (5'-GTTGGGTATATCACAATTTAT) and *IceA2R5* (5'-TTRCCCTATTTTCTAGTAGGT) yielded a fragment of 229, 334 or 439 bp according to the existence of repeated sequences of 105 nucleotides. PCR was performed under the following conditions: 4-minute preincubation at 94°C, followed by 30 cycles of 1 min. at 94°C, 1 min. at 53°C (both iceA1 and iceA2) and 1 min. at 72°C. Final extension was performed for 10 min. at 72°C. *iceA* amplimers were examined by electrophoresis on 1% agarose gels according to standard procedures [297].

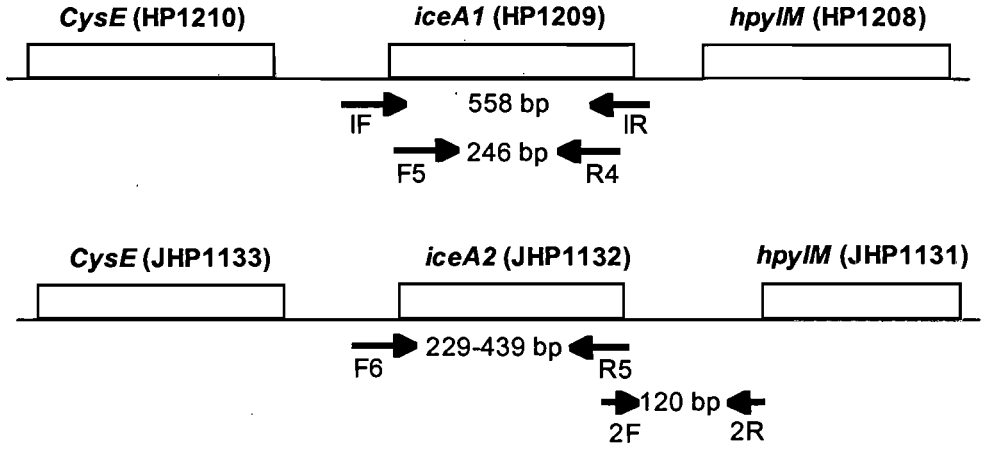


Figure 7.1 Primers, sites and amplicon sizes for *iceA1* (HP1209) and *iceA2* (JHP1132).

7.2.3 Sequencing of iceA1 and iceA2

iceA1 and *iceA2* were amplified using primer sets *IceAF5/R4* and *IceA2F6/R5*. PCR products were gel extracted (QIAEX II gel extraction kit, Qiagen, Cape Town, South Africa) and sequenced on an ABI PRISM 377 automated sequencer (ABI, Foster City, California, USA) using the ABI PRISM ™ BigDye ™ terminator cycle sequencing reagent kit with AmpliTaq ® DNA polymerase FS (PE Biosystems, Johannesburg, South Africa) as described previously [290]. PCR and direct sequencing were performed at least twice to determine DNA sequences for each strain.

7.2.4 Computer analysis

DNA and protein sequences were analyzed using the National Center for Biotechnology Information (NCBI) server (USA) and META Predict Protein (which includes the SignalP, JPRED, TopPred and DAS servers [Columbia University, USA]) [291,293,294,299]. SignalP predicts the presence and location of signal peptide cleavage sites, JPRED predicts secondary structures, TopPred is useful for the prediction of the location and orientation of transmembrane sequences through the use of hydrophobicity patterns and by applying the "positive-inside" rule while DAS predicts the location of transmembrane using hydrophobicity patterns. Internet-based searches were performed at NCBI, TIGR (Maryland, USA) and Astra-Zeneca (Boston, USA).

7.2.5 Statistics

Data (n = 109) were examined using the chi-squared test or Fisher's exact test as appropriate. Probability levels of < 0.05 were considered statistically significant.

7.3 Results

7.3.1 iceA1 sequence analysis

In a preliminary study, in order to establish the composition of *iceA* gene products in South African *H. pylori* isolates, *iceA1* (246 bp) and *iceA2* (229-334 bp) were sequenced.

Four isolates with the *iceA1* genotype (two from patients with gastritis alone and two from patients with peptic ulceration) were sequenced and compared to the predicted *iceA1* gene (HP1209) in strain 26695 (Figure 7.2). Alignment of these sequences revealed the presence of a putative conserved initiation codon at position ATG_{919} (GenBank Accession No. U43917), as previously reported [224] and no in-frame insertions or deletions were evident. All isolates shared substantial DNA (93-95%) homology with HP1209 over the 246 bp sequenced.

Figure 7.2 Alignment of *iceA1* nucleotide sequences showing the putative start codon.

```
HP1209:   TGTGTTTTTAACCAAAGTATCTGTGAAGACATTAAAAACCACTATAAGCAACAATGTTGT
Hp27:                        C
1715:                        C                          C           C
215:                         C                  TT      GC          C
464:                         C                  T

HP1209:   GCGATGTGTGGTGTGCGTGGCAACTCTGAAAACACTCAAATAGAGGTGGATCATAAAGAC
Hp27:           C    C T C                          A
1715:                C   G
215:                 C
464:                                                               T
          START
HP1209:   GGCCGCAAGGATGATTCAAGAGTTTCTGATTTAAGCACACAAGCTTTTGATGATTTCCAG
Hp27:                                        A G    GA             T
1715:                  C                            GA             T  A
215:                    T                           GA             T  A
464:        T     AA     C                    AT     GA             T

HP1209:   GCTTTATGTAAGCTTGTAATGATAAGAAACGCCAGATTTGTAAAAAATGCAAAGAGACTGGC
Hp27:           C             C                     G          A
1715:                         C                     G          A
215:                       C  C                     G          A
464:                          C                     G          A
```

All isolates therefore generated predicted proteins of 63 amino acids (from the consensus start), within the putative ORFs (Figure 7.3). The protein homology between HP1209 and the four South African isolates over this distance was 93-96%.

Figure 7.3 Putative proteins from the *iceA1* PCR fragments

```
HP1209:   MCGVRGNSENTQIEVDHKDGRKDDSRVSDLSTQAFDDFQALCKACNDKKRQICKKCKETGYR
Hp27:     MCGACGNSENTQIEVDHKDGRKDDSRVSDLSAQAFDDFQALCKACNDKKRQICKECKETGYR
1715:     MCGARGNSENTQIEVDHKDGRKDDPRVSDLSTQAFDDFQALCKACNDKKRQICKECKETGYR
215:      MCGARGNSENTQIEVDHKDGRKDDLRVSDLSTQAFDDFQALCKACNDKKRQICKKCKETGYR
464:      MCGVRGNSENTQIEVDHKDGRKNDPRVSDLNTQAFDDFQALCKACNDKKRQICKECKETGYR
```

7.3.2 iceA2 sequence analysis

Seven isolates with the *iceA2* genotype (two from patients with gastritis alone, two from patients with peptic ulceration and three from patients with gastric adenocarcinoma) were sequenced and compared to the predicted gene (JHP1132) present in the same locus in strain J99 (Figure 7.4).

Figure 7.4 Pile-up of *iceA2* genotypes. Peptide domains and sizes are indicated.

```
JHP1132:    MAVVIKVVNGKIQE YENGNYKRTYDSN IVAADTDGHIVAAVKTA
Hp27:       MAVVIKVVNGKIQE YENGNYKRTYGSN******************
1715:       MAVVVKVVNGKIQE FENGSHKRTYGSN******************
215:        MAVVIKVVNGKIQE YENGNYKRTYGSN******************
464:        MAVVIKVVNGKIQE YENGIYKRTYGSN IVAADTDGHIVAAV*TA
Ca102:      MAVVIKVVNGKIQE YENGNYKRTYGSN IVAADT*GHIVAAV*TA
Ca1104:     MAVVIKVVNGKIQE YENGIHKRTYGSN IVAADTDGHIVAAV*TA
Ca1506:     MAVVIKVVNGKIQE YENGIHKRTYGSN IVAADTDGHIVAAV*TA
                  14             13             16

JHP1132:    ************************************ KGKVEE
Hp27:       **********************AVNVQVSGGVVAVTTS KGKVEE
1715:       **********************AINVQVSGGVVAVTTS KGKMEE
215:        **********************AVNVQVSGGVVAVTTS KGKVEE
464:        KGKVEE FENGSYKRTYGSN AINVQISGGVMAVTTS KGKVEE
Ca102:      KGKVEE FENGSYKRTYGSN VINVQISGGVMAVTTS KGKVEE
Ca1104:     KGKVEE FENGSYKRTYGSN VINVQISGGVMAVTTS KGKVEE
Ca1506:     KGKVEE FENGSYKRTYGSN VIN*QISGGVMAVTTS KGKVEE
               6         13              15           6

JHP1132:    ************* ************************YKNGIHKRTY
Hp27:       ************* ************************YKNGSYKRTY
1715:       YKNGSYKRTTKRA NNNVQISGGVMAVTTS KGKVEECKNNGIHKGTY
215:        ************* ************************YKNGIHKRTY
464:        ************* ************************YKNGIHKRTY
Ca102:      ************* ************************CKNGIHKRTY
Ca1104:     ************* ************************CKNGIHKRTY
Ca1506:     ************* ************************CKNGIHKRTY
                  13              16          6        10
```

The *iceA2* gene from strain J99, JHP1132, conforms to the structure of repeats (14-13-16_{2B}-6-10) demonstrated by van Doorn [225] [224], generates a protein of 59 amino acids and is an example of the *iceA2B* subtype [224]. None of the South African isolates sequenced in this series had either a 2A (14-13-6-10) or a 2B motif. Two isolates (Hp27 & 215), however, had the sequence 14-13-16_{2C}-6-10 and could be classified as *iceA2C* subtypes. Four isolates had the sequence 14-13-16_{2B}-6-13-16_{2C}-6-10 and are examples of the *ice A2D* subtype. These subtypes typically generate proteins of 94 amino acids but one isolate (Ca102) had an in-frame 3 nucleotide deletion in 16_{2B} which resulted in a putative protein of 93 amino acids. Sequence analysis of a fifth isolate (1715) demonstrated a novel variant of the *iceA2D* subtype which was typified by the sequence 14-13-16_{2C}-6-13-16_{2C}-6-10 (sequence identity (71%) and positive residues (80%) with *iceA2D*).

Analysis of the protein signal sequences, secondary structures and topology for the South African *iceA2* variants is provided in Table 7.1.

Table 7.1 Protein characteristics of *iceA*-variants

		JHP1132	*HP27*	*Ca1506*	*1715*
Variant		2B	2C	2D	2D'
Amino acids (n)		59	59	94	95
#Cysteine (%)		0	0	0	1
#Proline (%)		0	0	0	0
Topology: N-terminus		"in"	"in"	"in"	"in"
Topology: C-terminus		?	?	?	?
TopPred	*PTM (n) +Position (Score)	None	None	One 58-78 (0.758)	One 23-41 (0.615)
DAS	PTM (n) Position:	None	One 32-41	Two 37-40 66-75	Two 31-41 70-76
#Helix (%)		11.7	0.0	0.0	0.0
#Sheet (%)		41.7	62.7	72.3	54.2
#Other (%)		46.7	37.3	27.7	45.8
#Class		Mixed	All-beta	All-beta	All-beta

"in" = orientation inside cell membrane, ? = not determined
*PTM = putative transmembrane segment, +Position = amino acid position, # from JPRED
DAS = Dense Alignment Sequence

Of note are the observations that these proteins do not have a typical prokaryote signal sequence (from the SignalP server), and the N-terminus appears to be embedded within the cell membrane. The TopPred topology program demonstrated that variants 2B and 2C had no predicted transmembrane regions whilst 2D and the novel 2D variant (2D') had a single putative transmembrane region with scores of 0.758 and 0.615 respectively (Figure 7.5) [293].

The Dense Alignment Surface (DAS) method confirmed that variant 2B had no transmembrane region but suggested a possible transmembrane region in 2C and confirmed two putative transmembrane regions in 2D and the 2D' variant [294]. Variant 2B can be classified as a mixed class protein, but 2C, 2D and 2D' are all *all-beta* proteins.

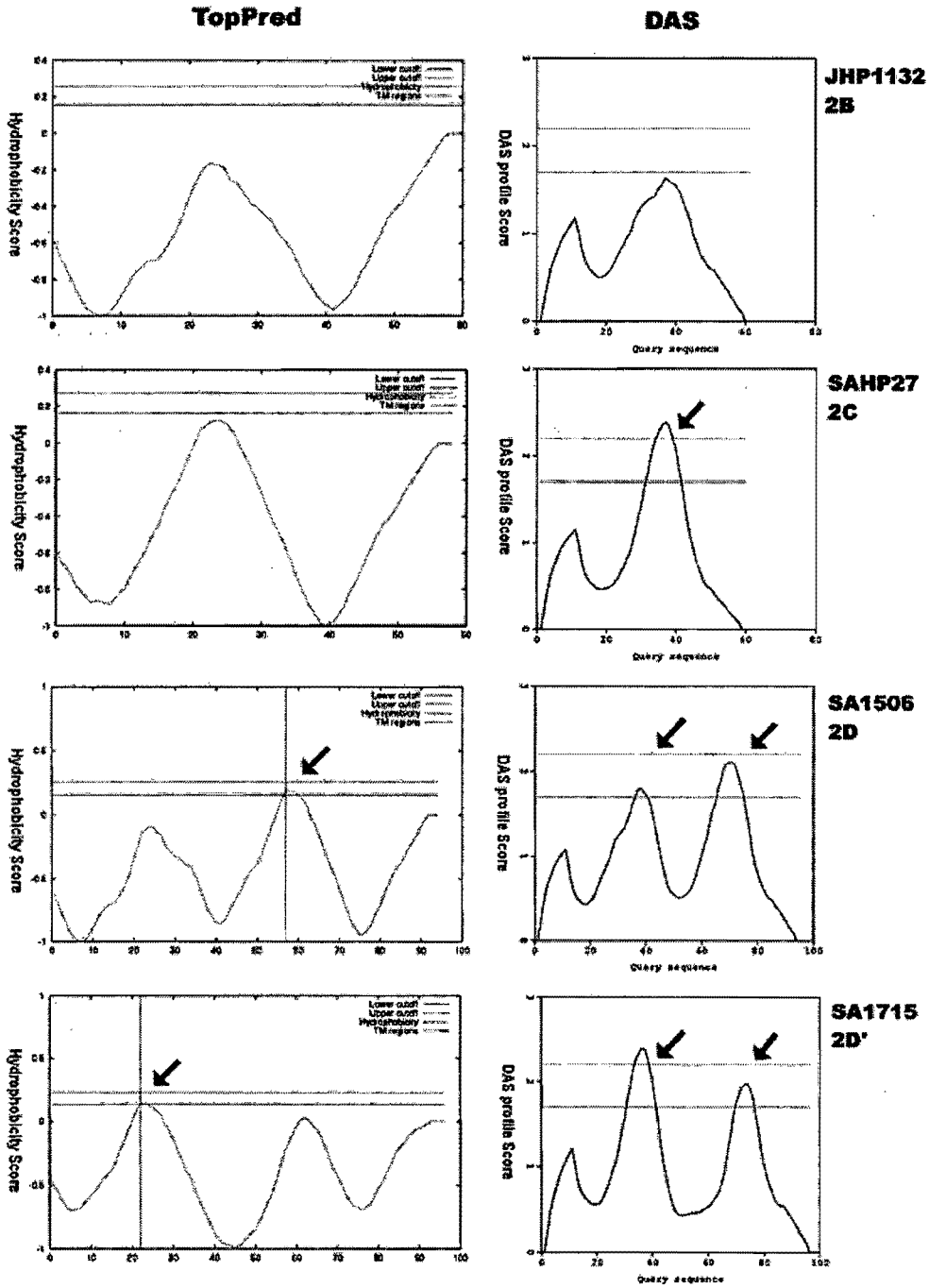


Figure 7.5 Results of topology profiles obtained using the TopPred program (left) and DAS (right) are shown as well as the isolates and the four *iceA2* variants (on the far right). Arrows indicate putative transmembrane regions.

7.3.3 Clinical relevance of iceA genotypes

After validating the PCR-protocol and examining the structure of *iceA1* and *iceA2*, PCR was performed in 109 defined clinical isolates and products were analyzed in terms of the distribution of genotypes. *iceA1* was detected in 74 (68%) of 109 isolates, and *iceA2* was found in 85 (80%). Fifty-four (50%) isolates were positive for both *iceA1* and *iceA2* using previously described primers *IceA1AF/1AR* and *IceA2F/2R* [12,223]. One isolate (1%) did not yield any PCR product for either of the *iceA* genotypes.

Twenty-one of the 54 *iceA1*$^{+}$/*iceA2*$^{+}$ isolates were re-examined with different *iceA* primer sets (F5/R4 and F6/R5) [185 224] to determine whether this mixed genotype was a consistent finding. All twenty-one isolates (100%) were positive for both genotypes, suggesting that these primer sets are concordant. These isolates each had a single *vacA* allele, a single *cagA*-3'-amplicon and were derived initially from single colony isolates. This suggests the presence of multiple *iceA* genotypes in these strains. PCR products from four isolates (HP27, 1715, 215, 464) were sequenced (cf. Chapter 7.3.1 & 7.3.2) confirming the presence of both alleles in these isolates.

Analysis of the distribution of *iceA* genes in all samples demonstrated a significantly higher prevalence of *iceA1* in isolates from patients with gastric adenocarcinoma (21 of 26 [81%]) than patients without clinically significant disease (27 of 47 [57%], p = 0.0376) (Figure 7.6A). Although the prevalence of *iceA1* was higher in isolates from patients with peptic ulcer disease (26 of 36 [72%]), this was not significantly different to gastritis alone (p = 0.123). Conversely, a significantly lower prevalence of *iceA2* alleles was found in the gastric adenocarcinoma group (16 of 26 [62%], p = 0.0029) than patients with gastritis alone (43 of 47 [91%]). Isolates from patients with peptic ulcer disease had intermediate levels of *iceA2* (29 of 36 [81%], p = 0.13 vs. gastritis alone, p = 0.086 vs. GCa).

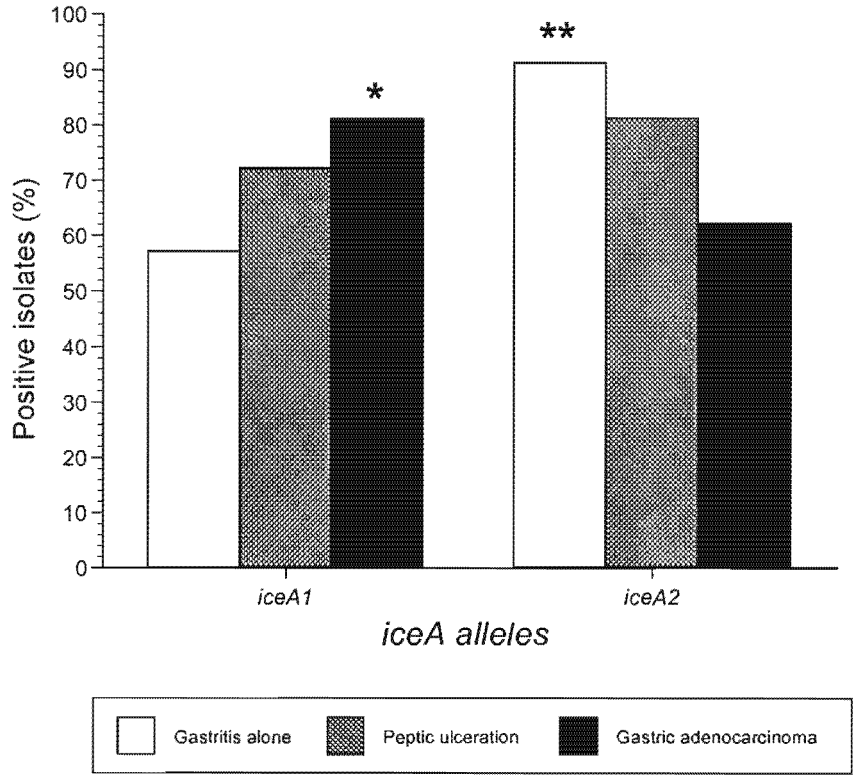


Figure 7.6A Distribution of *iceA* genotypes in all isolates. *p < 0.05 vs. gastritis alone, **p < 0.01 vs. gastric cancer.

Among the fifty-four isolates with a single *iceA* allelic type, the association between the distribution of the *iceA* genes (*iceA1* and gastric adenocarcinoma and *iceA2* and gastritis alone) was more striking ($p < 0.01$) (Figure 7.6B). No such relationship was evident for peptic ulcer disease ($p = 0.2$).

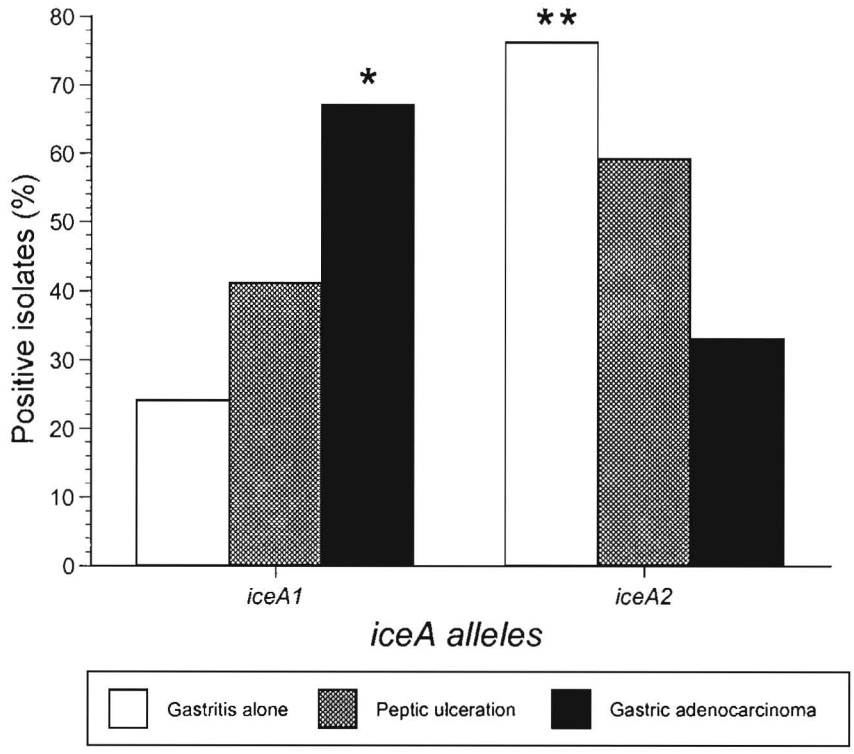


Figure 7.6B Distribution of *iceA* genotypes in 54 *H. pylori* isolates with single *iceA* genotypes. *$p < 0.01$ vs. gastritis alone, **$p < 0.001$ vs. GCa.

7.3.4 Distribution of iceA2 alleles

As previously reported [185] [224], most isolates with *iceA2* alleles (66 of 88 [75%]) could be divided into two types according to the presence of repeated sequences of 105 nucleotides and whether PCR products were ~229 bp (*iceA2B/C*) or ~334 bp (*iceA2D*) long. Twenty-two [26%] isolates had either both products or a combination of *iceA2B/C* and/or *iceA2D* or *iceA2E* (449 bp) products. These isolates each had single *vacA* alleles, and were derived from single colony isolates. This suggests the presence of multiple *iceA2* gene copies rather than DNA contamination.

The *iceA2B/C* subtype (229 bp) was predominant in gastritis cases (29 of 43 [67%], $p < 0.02$ vs. PUD) and was also found in GCa cases (11 of 16 [62%], $p < 0.03$ vs. PUD) (Figure 7.7). The *iceA2D* subtype (334 bp) was predominant in PUD cases (19 of 29 [66%], $p < 0.02$ vs. gastritis). The *iceA2E* subtype was more prevalent in GCa cases (7 of 16 [44%], $p < 0.04$) vs. gastritis). Among the sixty-six isolates with a single *iceA2* subtype, the association between the distribution of the *iceA2* subtypes (*iceA2B/C* and gastritis alone and *iceA2D* and PUD) remained statistically significant ($p < 0.003$).

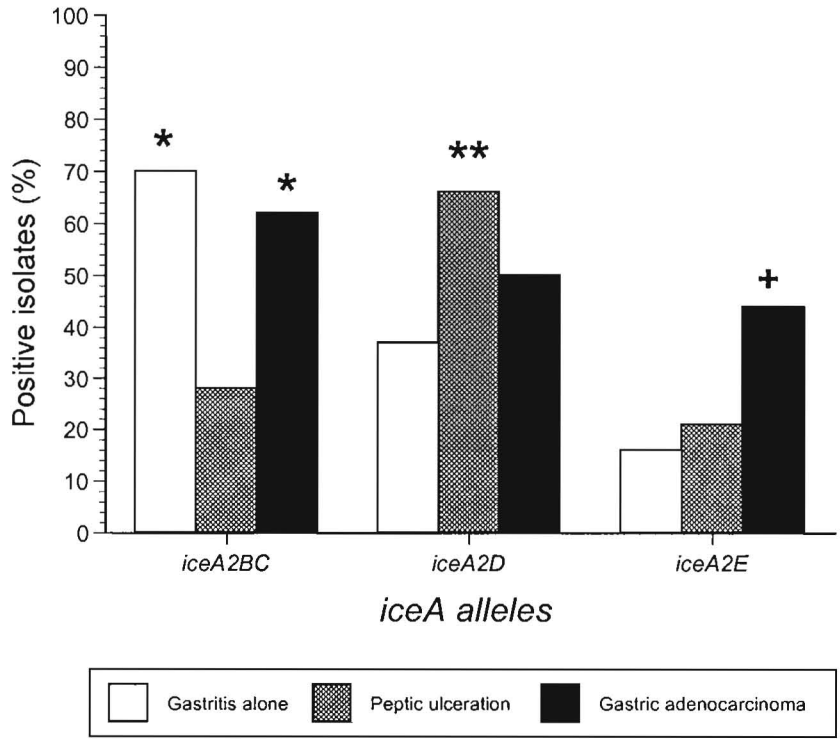


Figure 7.7 Distribution of *iceA2* subtypes and relation to disease. *$p < 0.01$ vs. PUD and GCa, **$p < 0.03$ vs. gastritis alone and GCa, $^{+}p < 0.04$ vs. gastritis alone.

7.3.5 Combination of iceA, vacA and cagA genotypes

All strains were *cagA*$^+$ and gave a single 3'-amplicon. By using the method of van Doorn et al. [185], eight different combinations based on analysis of the *vacA* s region (s1 and s2), and the *iceA* type (*iceA1*, *iceA2B/C* and *iceA2D* and *iceA2E*) were examined in patients with multiple genotypes (Figure 7.8). Significant associations between genotypes and clinical outcome were noted ($X^2 = 57.8$, $p < 0.000001$). Significantly more PUD isolates (69%, $p < 0.005$ vs. gastritis alone) and gastric adenocarcinoma isolates (81%, $p < 0.0005$ GCa vs. gastritis) were *vacA* s1/*iceA1*. In addition, significantly more PUD isolates were *vacA* s1/*iceA2D* (53%, $p < 0.0007$ vs. gastritis alone, $p = 0.07$ vs. GCa). In contrast, isolates from patients with gastritis alone were identified by either *vacA* s2/*iceA1* (21%, $p < 0.00001$ vs. PUD, $p < 0.0001$ vs. GCa) or *vacA* s2/*iceA2* (22%, $p < 0.00001$ vs. PUD, $p < 0.00003$ vs. GC).

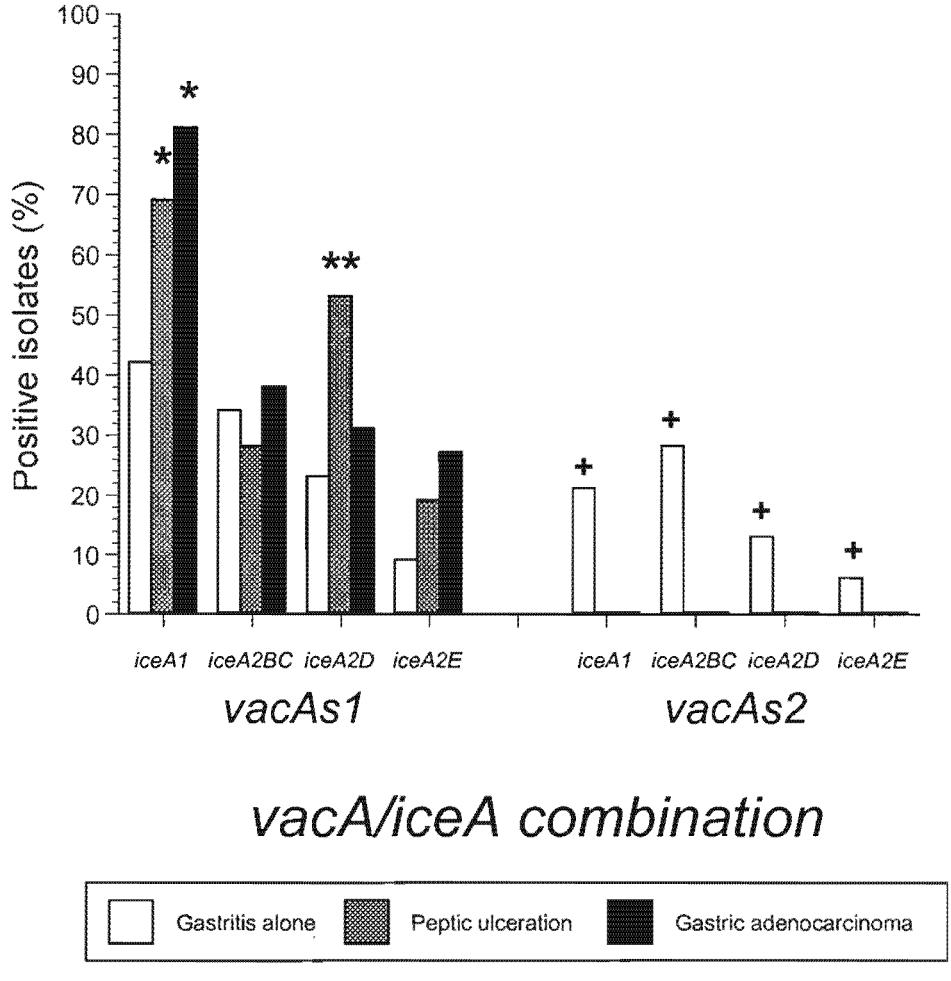


Figure 7.8 Combination analysis of *vacA/iceA* alleles and disease outcome in all 109 isolates. * $p < 0.01$ vs. gastritis alone, **$p < 0.005$ vs. gastritis alone, $^+p < 0.05$ vs. PUD or GCa.

In addition, a similar analysis was undertaken in patients with a single *iceA* genotype, with similar results (Table 7.2). Significant associations between genotypes and clinical outcome were noted (X^2 = 30.41, $p < 0.0008$). Significantly more PUD isolates (47%, $p < 0.01$ vs. gastritis) and GCa isolates (67%, $p < 0.0004$ vs. gastritis) were *vacA* s1/*iceA1*. In contrast, significantly more ($p < 0.009$) isolates from patients with gastritis alone were *vacA* s2/*iceA2BC*.

Table 7.2 Distribution of *vacA/iceA* alleles in single genotype isolates (n = 54).

Allele	**Gastritis** (n = 22)	**PUD** (n = 17)	**GCa** (n = 15)
vacAs1/iceA1	2 (19%)	8 (47%)*	10 (67%)$^{+}$
vacAs1/iceA2BC	5 (23%)	3 (18%)	4 (26%)
vacAs1/iceA2D	3 (14%)	6 (35%)	1 (7%)
vacAs2/iceA1	1 (5%)	0 (0%)	0 (0%)
vacAs2/iceA2BC	8 (36%)	0 (0%)**	0 (0%)**
vacAs2/iceA2D	3 (14%)	0 (0%)	0 (0%)

PUD = peptic ulcer disease, GCa = gastric adenocarcinoma

*$p < 0.02$ vs. gastritis, $^{+}p < 0.0008$ vs. gastritis, **$p < 0.006$ vs. gastritis.

7.3.6 Predictive value of vacAs1^{+}cagA^{+}cagPAI^{+}iceA1^{+} strains

Strains (*vacAs1*) with all the information encoding *cagA*, the *cag* PAI and *iceA1* were found in 21 of 36 (58%; $p < 0.0003$ vs. gastritis alone) peptic ulcer disease strains and in 14 of 26 (54%; $p < 0.003$ vs. gastritis alone) gastric adenocarcinoma strains compared to 9 of 47 (19%) strains from patients without clinically significant disease (gastritis alone). The sensitivity of *iceA1* as a marker for gastric disease (either GCa or PUD) based on the data generated in this study is ~50% (Table 7.3), while the specificity of *iceA1* is >80%. The predictive values for *iceA1* range between 61-70%.

Table 7.3 Predictive value of *vacAs1*$^+$*cagA*$^+$*cagPAI*$^+$*iceA1*$^+$ strains for clinically significant disease

Diagnosis	PPV	NPV	SE	SP
PUD	0.70	0.72	0.58	0.81
GCa	0.61	0.76	0.54	0.81

PUD = peptic ulcer disease, GCa = gastric adenocarcinoma

PPV = positive predictive value, NPV = negative predictive value, SE = sensitivity, SP = specificity.

7.4 Discussion

These findings support the hypothesis that strain differences in *iceA* genotypes may (partially) explain the differences in disease expression associated with *H. pylori* infection.

South African *H. pylori* isolates were distinguished by a higher prevalence of *iceA2* than *iceA1* alleles, as well as the large number of strains with both *iceA* genotypes. Fifty percent of organisms, irrespective of pathology, had both *iceA1* and *iceA2* genotypes. The reason for such a high prevalence of strains with both genotypes in South African patients appears to be a consistent finding since repeated PCR with different primers for *iceA1* and *iceA2* [185] on 21 of the 51 isolates consistently demonstrated both genotypes (*iceA1*$^+$/*iceA2*$^+$), and this was confirmed by sequencing. Nevertheless, it is possible that multiple strains might have been present in the culture from which the DNA was obtained. Mixed strains are commonly found and estimates range from 26% in a world-wide study [224], to 15% in the Netherlands [185], 4% in the USA [201], 22% in Colombia [201], and 17% in Japan [201]. However, single colony derivatives obtained by repeatable culturing of these "mixed" genotype strains produced putatively clonal strains which consistently produced a single *iceA* product [224].

Analysis of both "mixed" as well as single *iceA* genotypes demonstrated that although *iceA1* was more prevalent in PUD patients than in gastritis alone patients, this was not statistically significant. Previous studies in the USA [223] and the Netherlands [185], but not in South East Asia [201] have demonstrated a strong association between this allele and peptic ulcer disease. It is possible that the high prevalence (53%) of mixed *iceA* genotypes in patients with PUD may obscure any potential relationship between the allele and the disease. There was, however, a strong relationship between the combination of *vacA* signal sequence subtype, *iceA* alleles and peptic ulcer disease. Significantly more PUD isolates were *vacA* s1/*iceA1* compared to 40% of isolates from patients with gastritis alone.

The function of *iceA2* is unknown. In this study, amplicon size appeared to discriminate between peptic ulcer disease and gastritis alone. *iceA2BC* (229 bp) occurred in ~62% of isolates from South African patients with no clinically significant disease, while *iceA2D* (334 bp) was more prevalent (53%) in isolates from patients with PUD. Combination *vacA/iceA* analysis demonstrated that the *vacAs1/iceA2D* was more prevalent in patients with PUD (53%) than in patients with gastritis alone (23%). There seems to be a significant relationship between the cassette structure of *iceA2* and expression *in vivo*, where *iceA2ABC* variants are associated with higher mucosal transcript levels of this gene than *iceA2D* variants [300]. This suggests that down-regulation of *iceA2* expression, in addition to induction of *iceA*, may also contribute to disease outcome. It is possible therefore that South African strains from patients with clinically significant disease may not readily express *iceA2* since the majority of these isolates encode the *iceA2D* variant. Alternatively, alterations in the number of repeat cassettes results in potential changes in protein secondary structure. For example, variants 2A and 2B may exist as a single globular entity outside the cell, while variants 2C and 2D are predicted to display one and two surface exposed regions respectively. These changes may translate into differential binding and/or function of the protein. This has not been directly proven, but may result in "altered" pathogenicity of the organism.

There is a paucity of investigations of *iceA* alleles in isolates from patients with gastric adenocarcinoma. In the one small study to date, gastric cancer isolates from Japan and Korea were distinguished by the prevalence of *iceA1* (67%), while 75% of isolates from the USA were *iceA2* [201]. The numbers in this study were relatively small which limited statistical analysis. In a second Japanese gastric cancer study, *iceA1* was associated with enhanced gastric inflammation but not adenocarcinoma *per se* [301]. South African *H. pylori* cancer isolates, similar to South East Asia strains, were distinguished by the presence of *iceA1* and the absence of *iceA2*. In contrast, however, the prevalence of these alleles in cancer patients was significantly different to that found in patients without clinically significant disease in this study. In addition, gastric cancer isolates, unlike those from South African patients with PUD or gastritis were not distinguished by any specific *iceA2* allele. This data was strengthened when analyzed in combination with the *vacA* status of the strains. Significantly more gastric cancer isolates were distinguished by *vacA* s1/*iceA1* compared to patients without disease. The putative role for IceA1 in gastric cancer is not defined. Experimental studies have demonstrated that long-term colonization in both mouse and monkey models of experimental *H. pylori* infection require a wild-type *iceA1* strain [224,302]. It is possible that the higher percentage of *iceA1*$^{+}$ strains in South African gastric cancer patients may simply

reflect the fact they these patients have been infected for a longer time period. Alternatively, analysis of difference in *iceA1* gene transcription and therefore protein production may reveal a functional role for this protein in this condition [303].

The predictive value for type I strains has been noted (Chapter 6.3.4) and revising this classification to include strains which encoded all the information for the *cag*PAI served to increase both the positive predictive value as well as the specificity of this method as a predictor of clinically significant disease. Including whether the strains were *iceA1* or not further improved the positive value but weakened both the sensitivity and negative predictive value of any characterization. These alterations may be because of the relatively low (not significant) numbers of *iceA1*$^+$ isolates in patients with PUD. This is reflected in the decreased numbers of patients positive for all four markers (< 60%) compared to type I alone (100%) or type I + *cag*PAI (> 70%).

7.5 Conclusion

The sequences and prevalence of *iceA1* and *iceA2* in South African *H. pylori* isolates has been investigated. A novel *iceA2D* subtype has been identified. The potential association between *iceA1* and gastric adenocarcinoma, *iceA2D* and peptic ulcer disease and *iceA2C* and gastritis alone has been demonstrated in our study populations. No isolate from patients with clinically significant disease exhibited either the *vacA* s2/*iceA1* or the *vacA* s2/*iceA2* allele. Overall, this data support the hypothesis that there is a difference between organisms associated with disease and those not. An absolute separation cannot be made which suggests that other factors must play a role in disease pathogenesis. Analysis of *iceA* allelic types, however, is useful in South Africa and certain combinations of virulence factors may provide excellent negative markers for disease.

Chapter 8.

Analysis of genomic heterogeneity in *Helicobacter pylori* - relationship to gastroduodenal disease

8.1 Introduction

As discussed in detail (cf. Chapter 4), *Helicobacter pylori* is genetically one of the most diverse bacterial species so far reported [163]. It appears also subject to the highest known rate of intraspecific recombination [165]. The panmictic structure of the bacterial genome has been demonstrated [166], as has the possibility that it is clonal only over short time periods after natural transmission [165]. Genomic comparison has, however, demonstrated that the overall genomic organization, gene order and predicted proteins of two unrelated *H. pylori* isolates may be quite similar [42]. Variability extends to the last codon of the triplet (encoding bacterial amino acids); 20% of differences are found in this position [42]. Such information suggests that there might be a balance between panmictism and clonality within the organism. More recently, analysis of twenty *H. pylori* strains from diverse geographic regions indicate the finding of two weakly clonal groupings superimposed on a pattern of free recombination [167].

Several genome-based typing studies suggest that disease-specific strains of *H. pylori* may cluster together. Yoshimura et al. utilizing DNA-DNA hybridization have shown that strains from duodenal ulcer patients are more homologous to each other than to strains from patients with gastritis alone [169]. Repetitive extragenic palindromic (REP) sequences, which have been successfully used to differentiate bacterial strains associated with disease outbreaks [304-306], have been identified in *H. pylori* [170,307]. Cluster analysis of REP-PCR fingerprints from USA strains has demonstrated duodenal ulcer isolates to be more similar to one another than to isolates from patients with gastritis alone [170]. The ability of REP-PCR to identify virulence associated genes, however, appears to be limited [307]. Random-amplified polymorphic DNA (RAPD)-PCR has also been previously used to successfully differentiate clinical isolates [163,172] but have not been used in population based studies.

Analysis of specific genes demonstrates that the *cag* pathogenicity gene cluster and polymorphic types within *vacA* and *iceA* are associated with peptic ulceration and gastric cancer in South African populations while strains with the *vacA* s2 allele are usually found in patients with gastritis alone (cf. Chapters 5 & 6). The Cape-colored population is a mixed population descended mainly from East Africans, South East Asians (Indonesia), Indians

from Western India, native South Africans (mainly Khoisan) and Western Europeans [308], has the highest incidence of gastric adenocarcinoma in South Africa [156], has a high prevalence of *H. pylori* infection [4,5], and the organism is invariantly *cagA*$^+$ [297]. Sequence analysis of the *vacA* and *cagA* gene in *H. pylori* isolates from this population has suggested that these strains may be more clonal than German and Asian strains [165,167]. The question arises whether there is any correlation between virulence genotypes and strain classification in this population, and we therefore attempted to analyze isolate differences utilizing whole genome-based strategies.

8.2 Materials and Methods

8.2.1 H. pylori strains

A total of seventy-six clinical *H. pylori* isolates (20 gastritis alone, 34 peptic ulcer disease and 22 gastric adenocarcinoma isolates) were examined. *H. pylori* strain 26695 isolated from a patient in the UK with gastritis [41], and strain J99, isolated from a USA patient with duodenal ulcer disease [42] were used as positive controls.

8.2.2 PCR amplification typing of H. pylori isolates

For REP-PCR, the 18-mer degenerate primer pair REP1R-DT [304] and REP2-Dt [305] were used. Following initial denaturation of 95 °C for 3 min, each reaction consisted of 35 cycles of denaturation at 95 °C for 30 s., annealing and extension for 1 min. and final extension at 72 °C for 10 min. The annealing temperature was set at 45 °C. In additional experiments with 26695 and J99, PCR reactions were performed with a single primer to test the specificity of the product.

For RAPD-PCR, the specific *H. pylori* informative decanucleotide primer, 1254 (70%G+C) was used in a low-stringency PCR amplification [172]. Briefly, 4 cycles of 94 °C for 5 min, 36 °C for 5 min and 72 °C for 5 min were followed by 30 cycles of 94 °C for 1 min, then 36 °C for 1 min, and 72 °C for 1 min, with a final extension of 10 min at 72 °C.

Twenty microliters of each PCR mixture was electrophoresed through a 1% agarose gel with a standard of 100 bp or a 1kb DNA ladder (Roche Diagnostics, Johannesburg, South Africa). Variability in the intensity or shape of bands was not considered to represent differences. The signal sequence and the mid-region of the gene *vacA*, the *cagA* and *cag*PAI status and the *iceA* status of isolates was known (cf. Chapter 5,6, and 7).

8.2.3. Computer assisted analysis

Potential REP and RAPD-PCR sites in strain 26695 and J99 were identified using the NCBI blast server, and the expected number of band sizes identified for each. A cut-off of 10,000 bp for band sizes was chosen, based on PCR results.

The REP and RAPD-PCR fingerprints of the *H. pylori* strains were analyzed with GelCompar software Windows version 4.1 (Applied Math, Kortrijk, Belgium) [309]. Both REP and RAPD-PCR patterns were normalized using the 0.1 Kb pair molecular size standard. Comparison of the fingerprints was performed by Ward's method [310]. Strains were considered related when they shared banding patterns with a similarity coefficient > 99% [309].

For cluster analysis, the virulence data were summarized into two-way tables. Each table had 78 rows and columns for *vacA* genotypes (s1 or s2, m1 or m2), *cagA* 3'-fragment length (short [< 600 bp], medium [600-600 bp] or long [> 700 bp]), *cag*PAI (intact or partial), *iceA* status (*iceA1*$^+$, *iceA1*$^+$*2*$^+$ or *iceA2*$^+$), REP fingerprint (REP1 or REP2), RAPD fingerprint (RAPD1 or RAPD2) and disease classification (gastritis alone, PUD or GCa). The presence or absence of each character was binarily coded, present = 1, absent = 0. All analyses were performed with STATISTICA © software (Gaithersburg, Maryland, USA).

8.2.4 Statistics

Data were analyzed using the Wilcoxon rank sum test for independent samples, chi-squared test or Fisher's exact test as appropriate. Probability levels of < 0.05 were considered statistically significant.

8.3 Results

8.3.1 Potential REP sites in 26695 and J99

Initially, the expected number of REP-PCR products was identified in the two completely sequenced strains. NCBI BLAST analysis revealed a total of 480 possible bands (26695) and 742 bands (J99) respectively. Utilizing 10,000 bp as an upper limit (based on the PCR conditions), four and 8 PCR amplicons were predicted for 26695 and J99 respectively. REP-PCR was then performed on DNA isolated from each strain (Table 8.1). Repeated REP-PCR (n = 3) showed that fingerprints were stable.

Table 8.1 Expected and obtained REP-PCR products

26695		**J99**	
Expected	*Obtained*	Expected	*Obtained*
6,672	*5,604*	9,771	*2,130*
5,604	*2,720*	8,061	1,663
3,370	2,268	6,862	1,424
2,720	2,154	4,196	1,157
	1,424	3,815	1,044
	1,044	3,365	894
	806	*2,130*	806
	690	2,070	480
	480		
	371		

A large number of small amplicons (< 2000 bp) as well as the low frequency of predicted amplicons (20% for 26695 and 13% for J99) was demonstrated. The specificity of the REP products was then investigated by performing PCR with each primer separately. This generated nine bands for REP1R-Dt and 5 bands for REP2-Dt in 26695. Nine (64%) of the 14 bands were also found when both primers were used in a single reaction. In J99, nine bands for REP1R-Dt and 6 bands for REP2-Dt were identified. Three (20%) of the 15 bands were also found when both primers were used in a single reaction. Graphical analysis of the putative REP primer sites in both genomes demonstrated that both REP1R-Dt and REP2-Dt were not restricted to any specific region of the genome in either isolate. This data suggest

the possibility that the REP-PCR is largely non-specific and may instead be an arbitrarily primed protocol.

8.3.2 REP-PCR fingerprinting in clinical isolates

REP-PCR generated bands ranging in size from 0.1 to 6 kb pairs in all 76 isolates. Each isolate was distinguished by 1-9 distinct bands, with an average of 4 different bands per isolate. No amplification band was common to all strains. Repeated REP-PCR (n = 3) showed that fingerprints were stable. Of the 76 isolates, 58 different DNA fingerprints were seen; 2 pairs of isolates from the same patients and 6 pairs of isolates from different patients had DNA fingerprints with a similarity co-efficient > 99%.

Cluster analysis revealed that there was a large degree of genetic heterogeneity of the *H. pylori* strains in this study (Figure 8.1). Examination of the dendrogram demonstrated that REP-PCR divided isolates into two distinct and unrelated (similarity coefficient = 0%) clusters. Cluster 1 (REP1) consisted exclusively of 23 peptic ulcer isolates, 8 gastric adenocarcinoma isolates, and J99 which had been isolated from a patient with duodenal ulcer disease. No isolates from patients with gastritis alone were evident in this cluster. The second cluster (REP2) included 20 isolates from patients with gastritis alone, 13 gastric cancer isolates, 11 peptic ulcer isolates, and 26695, isolated from a patient without clinically significant disease. Statistical analysis demonstrated a significant difference in the distribution of isolates between these two clusters ($X^2 = 24.68$, $p = 0.000005$). Significantly more isolates associated with clinically significant disease were present in REP1 (100%) than in REP2 (55%, $p = 0.000002$).

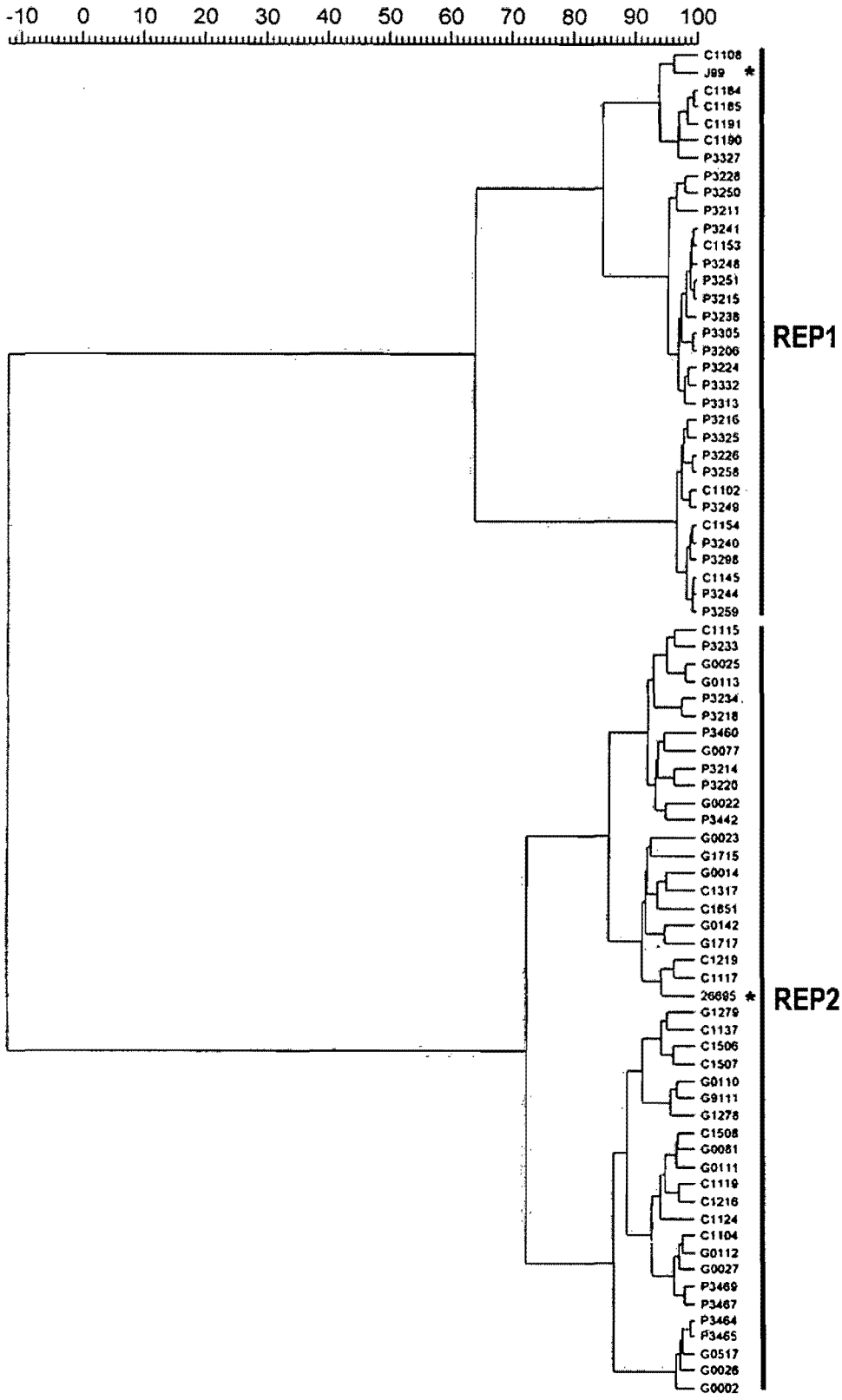


Figure 8.1 Dendrogram of *H. pylori* REP-PCR DNA fingerprints. Similarity coefficients are included in the top bar. G = gastritis alone, P = peptic ulcer disease, C= gastric adenocarcinoma. J99 and 26695 are indicated by asterisks. Two separate clusters are indicated.

8.3.3 Potential RAPD sites in 26695 and J99

The expected number of RAPD-PCR products were next identified in the two type strains. NCBI BLAST analysis revealed a total of 208 possible bands (26695) and 429 bands (J99) respectively. Two and 5 RAPD-PCR amplicons were predicted for 26695 and J99 respectively. RAPD-PCR was then performed on DNA isolated from each strain (Table 8.2). Repeated RAPD-PCR (n = 3) showed that fingerprints were stable.

Table 8.2 Expected and obtained REP-PCR products

26695		**J99**	
Expected	*Obtained*	Expected	*Obtained*
4,098	2,080	9,344	4,866
581	1,505	8,312	2,652
	1,098	5,725	*2,250*
	926	4,087	2,080
	727	*2,250*	1,843
	485		1,632
	254		1,134
			1,004
			698
			192

These results which include a large number of small amplicons (< 2000 bp) as well as the low frequency of predicted amplicons (0% for 26695 and 10% for J99) confirms that RAPD-PCR is, as described, an arbitrarily primed protocol. Graphical analysis of the putative RAPD primer sites in both genomes demonstrated that these sites were normally distributed.

8.3.4 RAPD-PCR fingerprinting

RAPD-PCR resulted in 1-7 distinct bands, with an average of 4 different bands per isolate. Repeated RAPD-PCR (n = 3) showed that fingerprints were stable. The band sizes ranged in size from 0.1 to 4.6 kb pairs, and there was no amplification band common to all strains. In RAPD-PCR of the 76 isolates, 60 different DNA fingerprints were seen; 5 pairs of isolates from the same patients and 3 pairs of isolates from different patients had DNA fingerprints with similarity co-efficients > 99%.

Cluster analysis revealed that there was a large degree of genetic heterogeneity of the *H. pylori* strains in this study (Figure 8.2). Examination of the dendrogram demonstrated 2 readily distinguishable clusters of *H. pylori* strains at a similarity coefficient of 64.2 ± 4.3%. Cluster 1 (RAPD1) included 5 isolates from patients with gastritis alone, 12 gastric cancer isolates and 17 peptic ulcer isolates. The second cluster (RAPD2) consisted of 17 peptic ulcer isolates, 10 gastric adenocarcinoma isolates and 15 isolates from patients with gastritis alone. Both type strains were also present in this cluster. Analysis suggested that the isolates associated with specific disease subtypes may not be differently distributed between the two clusters ($X^2 = 4.39$, $p = 0.111$). However, more isolates associated with clinically significant disease (both peptic ulcer disease and gastric adenocarcinoma) were present in RAPD1 (85%) then in RAPD2 (64%) ($p = 0.034$).

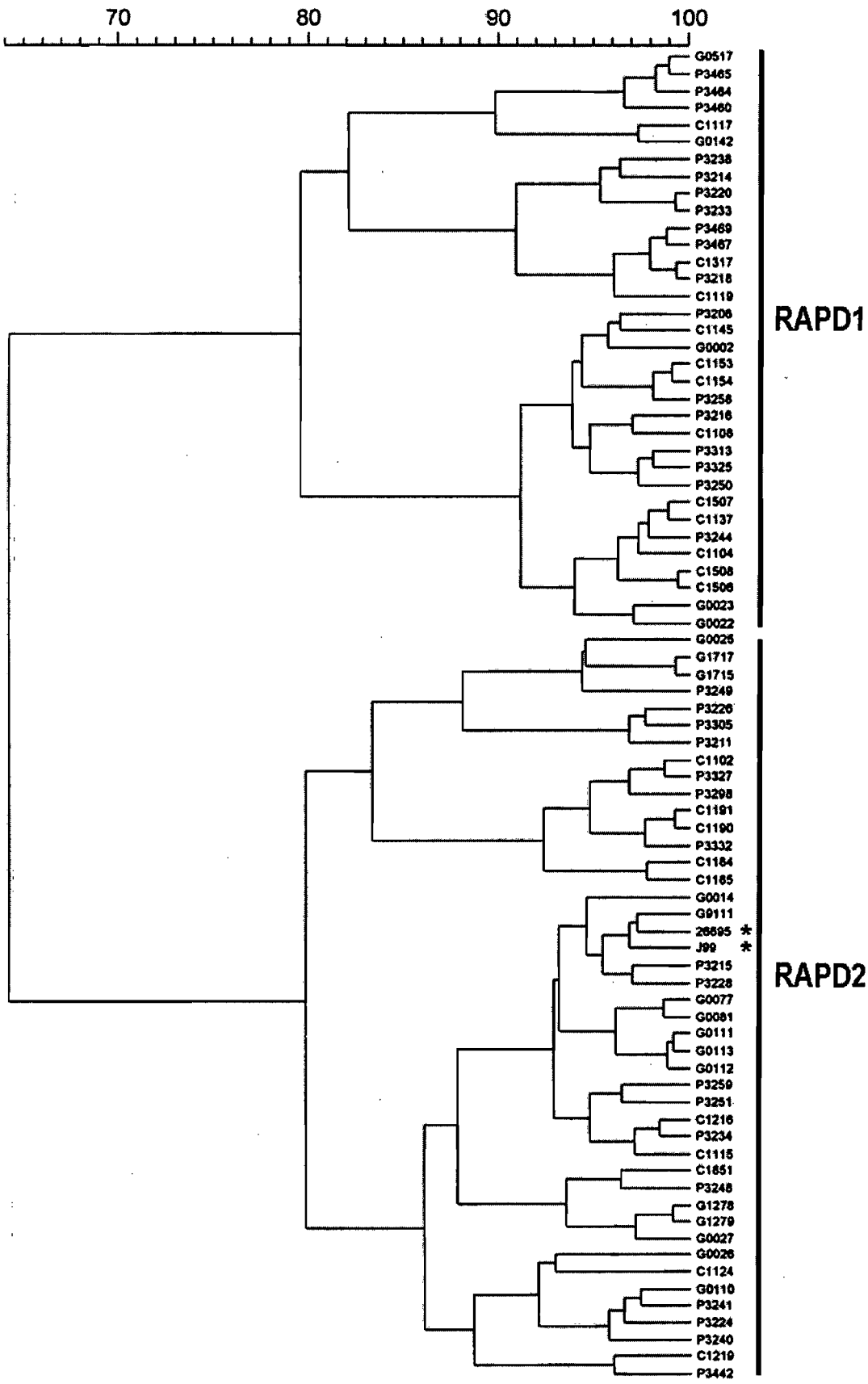


Figure 8.2 Dendrogram of *H. pylori* RAPD-PCR DNA fingerprints. Similarity coefficients are included in the top bar. G = gastritis alone, P = peptic ulcer disease, C= gastric adenocarcinoma. J99 and 26695 are indicated by asterisks. The two clusters are indicated.

8.3.5 Relationship between REP-DNA fingerprinting and virulence status

The dendrogram based on REP-DNA contains 2 clusters which segregate with pathology. As expected, analysis of *vacA* signal sequences demonstrated that *vacA* type s1 occurred more frequently in REP1 (100%), then in cluster 2 (77%, $p < 0.003$). There were also differences in segregation of *vacA* mid-regions between the 2 groups (88% vs. 61%, $p = 0.01$). No specific segregation of *cagA*-3'-fragment length or *cag*PAI status (intact or partial) was evident. *iceA* genotypes were, however, differentially segregated ($X^2 = 7.43$, $p < 0.03$). Specifically, *iceA1/2*$^+$ occurred more often in REP2 (62% vs. 33%, $p < 0.02$) while *iceA2* occurred more often in REP1 (42% vs. 18%, $p < 0.02$).

Within REP1, there were no differences in the distribution of either *vacA* s1 or *vacA* m1 between the different disease groups Twenty-eight (88%) of these isolates had the *vacA* s1m1 combination, while the two (9%) isolates from patients with peptic ulcer disease and two (22%) of gastric adenocarcinoma isolates had the *vacA* s1m2 combination. No differences in *iceA* genotypes were noted.

Within REP2, significantly more peptic ulcer disease isolates (100%, $p < 0.004$) and gastric adenocarcinoma isolates (100%, $p < 0.002$) were *vacA* s1, compared to gastritis alone isolates (50%). There were no differences, however, in the segregation of *vacA* mid-region alleles. No differences in *iceA* genotypes were noted.

8.3.6 Relationship between RAPD-DNA fingerprinting and virulence status

The dendrogram based on RAPD-PCR analysis contains 2 clusters which potentially correlate with pathology. Analysis of signal sequences demonstrated that *vacA* s1 type occurred more frequently in RAPD1 (100%), then in RAPD2 (77%, $p < 0.002$), while *vacA* s2 alleles were found exclusively in the second cluster. There were no differences in segregation of *vacA* mid-regions between the 2 clusters (74% vs. 70%, $p < 0.5$). No specific segregation of *cagA*-3'-fragment lengths was evident. The *cag*PAI status was, however, differentially segregated ($p < 0.002$). Specifically, an intact pathogenicity island was present more often in RAPD1 (85%) than in RAPD2 (52%). While *iceA* genotypes did not appear to be differentially segregated between the two clusters ($X^2 = 5.4$, $p = 0.066$), *iceA2* genotypes were present significantly more often ($p < 0.02$) in RAPD2 (63%) than in RAPD1 (15%).

Within RAPD1, all five isolates from patients with gastritis alone were *vacA* s1m1. There were therefore no differences in the distribution of *vacA* alleles between the different disease groups in this cluster (*vacA* s1 = 100% all isolates, *vacA* m1 = 71% peptic ulcer disease, 67% gastric adenocarcinoma, 100% gastritis alone). Neither *cag*PAI status nor *iceA*

genotypes ($X^2 = 5.86$, $p = 0.053$) segregated between the disease groups, but *iceA2* was more prevalent in peptic ulcer disease isolates (30%, $p = 0.05$) than gastric adenocarcinoma isolates (0%).

Within RAPD2, significantly more peptic ulcer disease isolates were *vacA* s1 (100%, $p < 0.00005$) and *vacA* m1 (94%, $p < 0.0005$) than gastritis alone isolates (s1=33%, m1 = 33%). This was similar for gastric adenocarcinoma isolates (*vacA* s1 = 100%, $p < 0.001$, *vacA* m1 = 80%, $p < 0.03$). No specific segregation of *iceA* genotypes was evident. The *cag*PAI status was, however, differentially segregated between disease groups in RAPD2 ($X^2 = 12.13$, $p < 0.003$). Specifically, an intact pathogenicity island was present more often in peptic ulcer disease isolates (78%, $p < 0.001$) and gastric adenocarcinoma isolates (60%, $P < 0.05$) than gastritis alone isolates (23%).

8.3.7 Cluster analysis of virulent and fingerprint data

Hierarchical analysis was performed using Unweighted pair-group average (UPGMA) and Euclidean distances for the variables. This resulted in two distinct clusters (Figure 8.3) which were clearly delineated. Cluster 1 was defined by the presence of gastritis alone and gastric adenocarcinoma isolates, *vacA* s2 and m2, a partial *cag*PAI, medium and long *cagA*-3' fragment lengths, the *iceA* alleles and the RAPD1/REP2 fingerprints. Further analysis of cluster 1, however, suggested the presence of 2 sub-clusters (1a and 1b). Cluster 1a demonstrated that gastritis, *vacA* s2, a partial *cag*PAI and the *iceA2* genotype were related, while cluster 1b was characterized by gastric adenocarcinoma isolates, the medium and long *cagA*-3'fragments and *iceA1*. The RAPD1 and REP2 fingerprints occurred outside these clusters.

Cluster 2 included PUD, REP1, the virulence associated *vacA* s1 and m1 alleles, an intact *cag*PAI, RAPD2 and a short *cagA* 3'-fragment length. This suggests that PUD was associated with specific virulence genes as well as specific genomic fingerprints. Clustering using the k-Means test confirmed the presence of two clusters. Using the latter analysis, cluster 2 contained, in addition, the $iceA1^+/2^+$ variable.

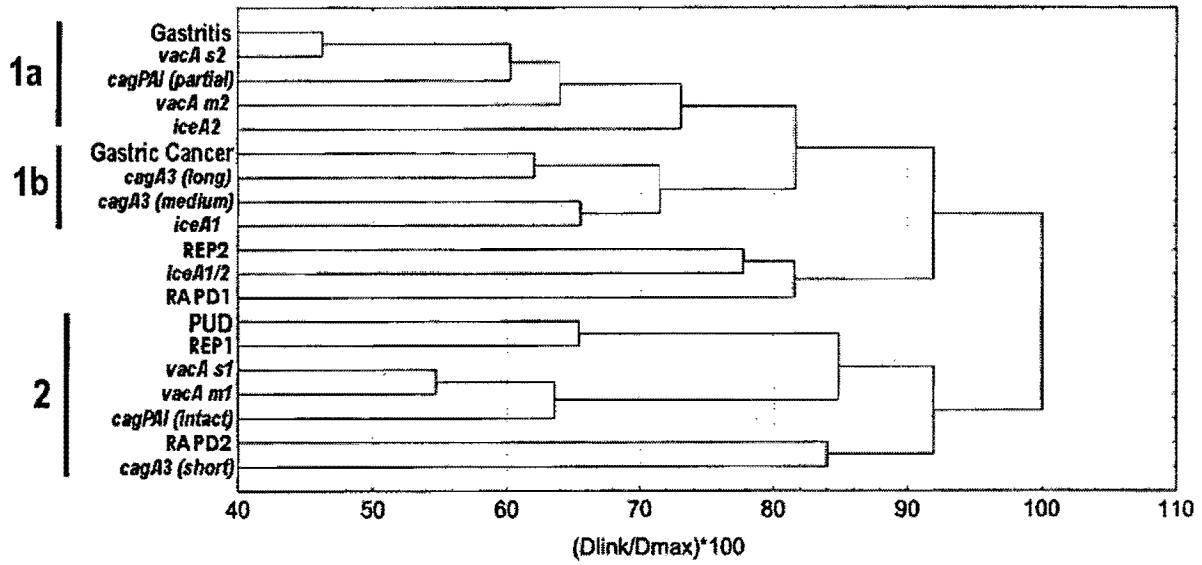


Figure 8.3 Cluster diagram based on hierarchical analysis using genomic analysis (REP and RAPD fingerprints), and virulence data (*vacA*, *cagA* and *cag*PAI, and *iceA* genotypes). The two major clusters are evident, as are the sub-clusters in I.

8.4 Discussion

The DNA fingerprints of 76 *cagA*$^+$ *H. pylori* isolates from Cape-colored individuals with a panoply of gastroduodenal diseases were analyzed by two complementary genomic DNA based polymerase chain reaction techniques, REP-PCR and RAPD-PCR. This study indicates that REP-PCR is probably an arbitrarily-primed protocol rather than a site-directed genome analysis. Nevertheless, because of differences in primers and annealing temperatures, these techniques will generate different fingerprints. Both the REP and the RAPD-PCR fingerprints were stable and reproducible for each isolate. It appears, however, that REP-PCR may be more useful than RAPD-PCR for generating DNA fingerprints that can classify pathogenic South African strains.

REP-PCR typing has generated stable, reproducible DNA fingerprints which have been successfully used to discriminate *H. pylori* strains associated with peptic ulcer disease in a USA study [170]. The results from the present study suggest that REP fingerprints (although probably generated as arbitrarily primed products) may be useful for defining disease-associated strains. In particular, REP-PCR clearly discriminated a pathogenic cluster which accounted for 69% of peptic ulcer disease isolates and 41% of gastric adenocarcinoma isolates in our study group. This cluster was also distinguished by the almost universal presence of the *vacA* s1m1 allelic type, as well as the inclusion of the type strain (J99, *vacA*

s1bm1) isolated from a patient with duodenal ulcer disease. The *vacA* s2 allele (low or non-cytotoxic activity, not associated with peptic ulceration in the USA [11]) was completely absent from this cluster. The second REP cluster was composed chiefly of gastritis alone isolates (61%). In addition, *vacA* s2 alleles were found exclusively in this cluster, while the *vacA* m2 allele, which has been less strongly correlated with epithelial injury (epithelial degeneration, mucus depletion and microscopic erosions) than mid-region m1 [6] tended to segregate into this cluster (81%). Interestingly, a fair percentage of gastric adenocarcinoma isolates (59%) was also found in cluster 2. These results suggest that carcinogenic potential, unlike that for peptic ulcer disease, may not be associated with any particular REP cluster. Interestingly, *iceA* genotypes appeared to segregate with REP fingerprints. Specific subtypes of *iceA2* did not, however, separate between the two clusters. Both *iceA2C* and *iceA2D* were equally present in REP1 while a mixed genotype (*iceA1/2*$^{+}$) was principally found in REP2. The latter was often characterized by the *iceA2D* subtype (60%) which identifies isolates from patients with PUD (cf. Chapter 7).

RAPD-PCR uses an oligonucleotide of arbitrarily chosen sequence to prime DNA synthesis from pairs of sites to which it is matched or partially matched. It results in strain specific arrays of DNA products and has been successfully used to analyze DNA diversity among clinical isolates of *H. pylori* [172,180]. The RAPD results from this study using a high (70%) GC content primer show two main clusters, one containing mainly isolates from patients with peptic ulcer and gastric adenocarcinoma (RAPD1) and the other isolates mainly irrespective of pathology (RAPD2). In contrast to REP fingerprinting which distinguished peptic ulcer isolates into a separate cluster, RAPD-PCR may not be associated with peptic ulcer disease potential, isolates from peptic ulcer disease patients being present in similar numbers in both RAPD1 and RAPD2. Similarly to REP fingerprinting, there was a correlation between RAPD-PCR and *vacA* polymorphism. Strains with the *vacA* s2 allele were exclusively present in isolates from RAPD2, while RAPD1 contained only strains with *vacA* s1 alleles. As noted for REP analysis, the *iceA2* genotype segregated with RAPD fingerprints. Interestingly, an intact *cag*PAI also appeared to segregate with the fingerprint (RAPD1).

Sixty-four percent of strains identified in the virulence-associated REP1 were also present in the heterogeneous RAPD2 (but made up only 48% of isolates identified in this cluster), suggesting that the RAPD and REP techniques may be only partially complementary. It would appear that REP-PCR and RAPD-PCR differently differentiate groups of organisms. The former identifies isolates associated with clinically significant

disease, and that carry virulence associated genes, *vacA* s1m1 and *iceA2*. The resultant is a clearly defined virulence group. The latter technique, however, appears to segregate isolates based on virulence genes but not consistently by pathology. In addition, this is obscured by the complex nature of RAPD2 where the association between virulence genes (*vacA* s1m1 and an intact *cag*PAI) and PUD is diluted by the presence of non-virulent genes and a high prevalence of isolates from patients with gastritis alone. It is possible that this may merely reflect a statistical anomaly. It should also be pointed out that these results are only indicative of a particular time in the natural history of infection and it is feasible that with time patients with gastritis alone whose isolates are identified in RAPD2 may develop clinically significant disease.

Alternatively, such anomalies may be as a result of the methodology used (Ward's method using the GelCompar program). Binary coding and hierarchical analysis using UPGMA and k-Means clustering with the STATSITICA program demonstrated a clear clustering of PUD, virulence associated *H. pylori* alleles (*vacA* s1m1 and an intact *cag*PAI) and the REP1/RAPD2 fingerprint. This was clearly delineated from a second cluster containing the remainder of the variables, and did not generate the anomalies noted with the GelCompar program. This alternative methodology may potentially provide a more robust method of analyzing the relationship between the genome and virulence. Interestingly, this result differs to that of Salaun et al. whose similar analysis of the genome and virulence factors demonstrated a panmictic structure of *H. pylori* [166]. It is feasible that the relationships noted in the present study reflect the possibility that *H. pylori* in the Cape-colored population may be more clonal than European strains [165].

8.5 Conclusion

Analysis of genome relatedness demonstrated that computer-assisted analysis of clustering of REP-PCR fingerprints was strongly associated with disease and with *vacA* signal sequence type in South African isolates from Cape Town, while RAPD-PCR fingerprints were less strongly associated with these parameters. Isolates from peptic ulcer patients and patients without ulcers tended to cluster differently, but strains from patients with gastric adenocarcinoma appeared to show no specific genomic clustering. This is novel, particularly because only one other study has shown *H. pylori* genome-relatedness and gastric disease clustering. Additional studies using a number of molecular genetic techniques in other South African population groups as well as in geographically isolated groups require to be conducted to explore the results from this South African population.

Chapter 9

Identification and analysis of the effect of *H pylori* LPS on naïve ECL cells

9.1 Introduction

In the preceding four experimental chapters, potential virulence factors in *H. pylori* isolates from the Western Cape were analyzed and their relationship to pathology defined. These results suggest strongly that clinically significant gastroduodenal diseases in South Africa are partially due to the virulence of the infecting organism.

Infection with the organism (irrespective of virulence), however, results in a number of endocrine and physiological alterations in the gastric mucosa (reviewed more fully in Chapter 3). These effects may be modulated by LPS. Essentially, *H. pylori* can elevate plasma gastrin levels [311-313] and increase acid output in patients compared with uninfected persons [72,73]. In addition, whilst serum gastrin levels are similar in *H. pylori* infected healthy volunteers and patients with duodenal ulcer disease, acid secretion is disproportionately increased in patients with the disease [314]. The effects of *H. pylori* on acid secretion appear to depend both on the stage of bacterial infection, and have been reported as diminished, normal or increased [72,73,315].

Although the relationship of *H. pylori* infection and gastrin and somatostatin (SST) cell function has been studied extensively [72,315], little is known of the effects of this infection on the fundic enterochromaffin-like (ECL) cell. This neuroendocrine cell, which is critical in the regulation of parietal cell secretion, is both the major source of histamine in the mucosa [316] and a prime target for the secretory and trophic effects of gastrin [317]. Although the immediate effect of gastrin is to initiate synthesis and secretion of histamine, its long-term action is to promote ECL cell hyperplasia and possibly even neoplastic transformation, particularly in rodents [318]. The effects of *H. pylori* on ECL cell proliferation or histamine secretion have not been reported.

One may postulate that *H. pylori* might affect acid secretion by altering the histamine secretory response of the ECL cell. To investigate this question and to explore the possible relationship between *H. pylori* infection and ECL cell hyperplasia, the effects of *H. pylori* LPS on either histamine secretion or DNA synthesis were examined in rat ECL cells.

9.2 Materials and Methods

9.2.1 Experimental design

An isolated, purified preparation of rat ECL cells maintained as a primary short-term cell culture was used in all experiments [319]. The effect on ECL cell histamine secretion over 1 hr of both *H. pylori* and *E. coli* LPS (10 fg/ml – 10 ug/ml) alone or with maximal or sub-maximal concentrations of gastrin (10^{-8} M or 10^{-10} M) was initially examined. To investigate whether *H. pylori* LPS-mediated histamine secretion occurred via activation of the gastrin/CCK_2 receptor the effect of adding the specific receptor antagonist, L365,260 (10^{-12} – 10^{-6} M) was evaluated. Thereafter, the effect of the ubiquitous gastrointestinal inhibitor somatostatin (10^{-16} – 10^{-6} M), on LPS-stimulated histamine secretion was determined. Cells were initially pre-incubated with either L365,260 or somatostatin for 30 min, followed by 1 hr incubation with *H. pylori* LPS (100 ng/ml). The presence of the CD14 receptor was thereafter identified by reverse transcription-polymerase chain reaction (RT-PCR) from cDNA derived from ECL cells. Subsequently, the effect of *H. pylori* and *E. coli* LPS (10 fg/ml – 10 ug/ml) alone or in combination with a maximal dose of gastrin (10^{-8} M) on DNA synthesis by measurement of the 24 hr uptake of bromodeoxyuridine (BrdU) was investigated.

9.2.2 Materials

All materials were obtained commercially from Sigma Chemical Co. (St. Louis, Missouri, USA) except where otherwise indicated. Lipopolysaccharide (LPS), prepared from the cytotoxic ($VacA^+/CagA^+$) *H. pylori* strain 84-183 by hot phenol-water extraction exactly as previously described [320], had a molecular weight of 5,000-10,000 Daltons. *E. coli* LPS, (Sigma Chemical Co.) was prepared from cells of serotype 026:B6 by phenol extraction and ion-exchange chromatography; the RNA and protein concentrations were < 1%.

9.2.3 Buffers

The composition of medium A (mM): NaH_2PO_4 (0.5), Na_2HPO_4 (1.0), $NaHCO_3$ (20), NaCl (70), KCl (5), glucose (11), Na_2-ethylenediaminetetraacetic acid (EDTA) (1.0), HEPES buffer (50) at pH 7.8, and BSA (10 mg/ml). Medium B was similar to A except for $CaCl_2$ (1.0) and $MgCl_2$ (1.5) at pH 7.4 and there was no EDTA. Medium C contained HEPES buffer (15) (pH 7.4), NaCl (140), $MgSO_4$ (1.2), $CaCl_2$ (1.0), D-glucose (11), BSA (1 mg/ml), dithiothreitol (0.5). Stock Nycodenz: $MgCl_2$ (1.2), HEPES buffer (15), BSA (10 mg/ml), Nycodenz (Accurate Chemical and Scientific Corporation, Westbury, New York, USA). Stock Nycodenz was diluted 1:1 and 1:2 with medium C. 6 ml of 1:1 solution was overlaid by 7 ml of 1:2 solution to form the step gradient for ECL cell purification.

9.2.4 Cell isolation

Rat ECL cells were prepared by a modification of the method of Prinz, as previously described [319]. For each experiment, 4 non-fasting female Sprague-Dawley rats (200 - 250g) were sacrificed by cervical dislocation subsequent to CO_2 anesthesia. The stomachs were excised and everted to mucosal-side-out sacs. Following a thorough wash in phosphate buffered saline (PBS), pronase E solution (Boehringer Mannheim, Indianapolis, Indiana, USA) (1.6 mg/ml) was injected into the stomach sacs (3 ml). The stomachs were then incubated in oxygenated medium A for 30 min at 37°C, followed by a 10 min incubation in a calcium respiration medium (B) at the same temperature. The stomach sacs then were transferred back to medium A for a further 30 min incubation. The mucosal cells were harvested by gentle stirring for 10 min in medium B and collected by centrifugation (100 x g, 5 min).

9.2.5 Cell purification

The crude mucosal cell fraction was subjected to counter-flow elutriation (JE-6 rotor, Beckman Instruments). Small cells with a diameter of 8-10 µm were collected at 2,000 rpm and a flow rate of 20 ml/min. This fraction was subsequently layered on a step Nycodenz gradient and centrifuged at 1100 rpm for 8 min. The ECL cell-enriched fraction (90-95%) was collected by aspiration at the interface of density 1.040 g/ml. After washing in Medium C, the viability and number of this enriched fraction was evaluated by trypan blue exclusion.

9.2.6 Short term culture

The enriched ECL fraction was resuspended in growth medium (Dulbecco's Minimal Essential medium (DME):F12, 2% Fetal Calf Serum (FCS), Insulin Transferrin Sodium Selenite 0.5 mg/ml, hydrocortisone 10 nM, gentamicin 0.1 mg/100 ml, pH 7.4) at a final concentration of 10^5cells/ml. Thereafter, 100 µl of cell suspension was applied per well in rat collagen-I coated 96-well plates (Collaborative Research, Bedford, Massachusetts, USA) for measurement of DNA synthesis. For measurement of histamine secretion, 200 µl of cells were applied per well in a 48-well plate and incubated overnight.

9.2.7 Histamine secretion

Measurements of basal and gastrin (human gastrin-17, $1x10^{-8}$ M) stimulated histamine release were undertaken to confirm cell function and viability. In a previous study the EC_{50} for gastrin was determined to be 10^{-10} M and the maximal response 10^{-8} M [319]. In all experiments, growth medium was removed and replaced with 200 µl of culture medium (DME-F12, 2% FCS, pH 7.4) containing agents of interest. Control wells contained culture medium alone. At

the termination of the experiment, 180 µl of medium was removed and, after centrifugation at 10,000 rpm, decanted and stored at -20°C for subsequent histamine measurement. Histamine analysis was performed by enzyme immuno-assay (AMAC, Immunotech, Westbrook, Maine, USA) and expressed as nmoles/10^3 cells. Final results were expressed as mean percentage of control ± SEM.

9.2.8 RT-PCR

The FastTrack mRNA isolation kit (Invitrogen, San Diego, California, USA) was utilized to isolate mRNA from ECL cells. SuperScript preamplification system (Gibco BRL, Gaithersburg, Maryland, USA) was utilized to prepare first strand cDNA. Specific DNA amplification for CD14 was carried out with the following combinations of primers derived from the CD14 nucleotide sequence [268]: sense: 5'-CTTGAACCTCCGCAACGTGTC and antisense: 5'-CCCAGTGAAAGACAGATTGA. PCR using 0.7 µg cDNA template was carried out with Taq DNA polymerase through 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 45°C) and extension (1 min at 72°C) on a thermal cycler from MJ Research (Watertown, Massachusetts, USA). Products were electrophoretically separated and visualized using UV.

9.2.9 DNA synthesis

Cells were cultured overnight for analysis of DNA synthesis. Subsequently, the medium was aspirated and replaced with growth medium containing BrdU (pre-diluted labeling reagent (1:200) – Amersham, Arlington Heights, Illinois, USA) and the indicated stimulant. After incubation for a further 24 hr, the medium was removed by inverting the plate, the cells fixed at 4°C in 90% ethanol/5% acetic acid/5% water, then washed in PBS/Tween-20 (0.1%). The wells were incubated with anti-BrdU antibody with nuclease (1:400 - Amersham) for 1 hr at room temperature, and following 3 washes, incubated with rabbit anti-mouse IgG conjugated to horseradish peroxidase (1:800 - Amersham) for 30 min at room temperature. After further 3 washes, wells were incubated with substrate (30 mg O-phenylenediamine dihydrochloride in citrate-phosphate buffer, pH 4.12), and optical density determined spectrophotometrically at 405 nm (Biorad Microplate reader model 450, Winooski, Vermont, USA). Results are expressed as OD $\text{units}_{(405\ nm)}$ and final results are expressed as mean ratio to control ± SEM.

9.2.10 Analysis of cytotoxicity

To assess non-specific damage to the ECL cells, the presence of lactic acid dehydrogenase (LDH) in culture supernatants was assessed. LDH was measured in overnight cultured samples stimulated for 1 hr with either *H. pylori* or *E. coli* LPS (both 10 ug/ml). Final results

are expressed as percentage of control levels (LDH release from unstimulated cells). In addition, freshly isolated ECL cells (5 x 10^4 cells) were incubated at 37°C for 0, 5, 15, 30, 60, 90, 120 min or 24 hr with either *H. pylori* or *E. coli* LPS (both 10^{-6} M) in PBS and cell viability was assessed by trypan blue exclusion. Final results, representing viability in PBS, are expressed as percentage of control levels.

9.2.11 Statistical analysis

Results are expressed as mean ± SE. "n" indicates the number of separate ECL cell preparations. Statistical analysis was performed using the two-tailed Student's t-test for paired values as appropriate and p-values < 0.05 were considered significant.

9.3 Results

9.3.1 Effect of E. coli LPS on Histamine secretion

Initially the effect of *E. coli* LPS on basal and stimulated ECL cell histamine release was measured. Basal levels for the rat ECL cell preparation were 3.4 ± 0.13 nmol/10^3 cells. Although gastrin (10^{-8} M) stimulated histamine release as expected (Figure 9.1), *E. coli* LPS alone did not significantly alter basal histamine release over the 1 hr experimental period (Figure 9.1). However, co-incubation of *E. coli* LPS (10 ug/ml) resulted in a significant inhibition of gastrin-stimulated histamine secretion. These results are consistent with early *in vivo* data [321], the mechanism is, however, unknown.

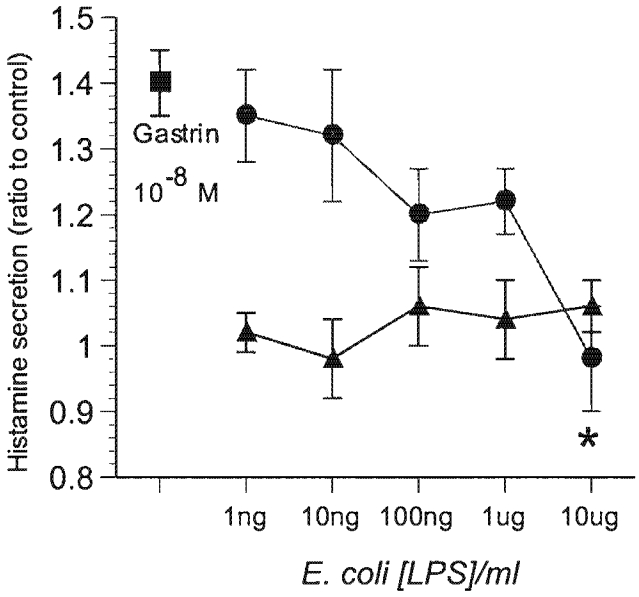


Figure 9.1 Effect of *E. coli* LPS on ECL cell histamine secretion. ■ Gastrin 10^{-8} M, ● LPS + gastrin (10^{-8} M), ▲ LPS alone. All points are expressed as means ± SEM. *p = 0.03 (n = 5).

9.3.2 Effect of H. pylori LPS on Histamine secretion

The effect of *H. pylori* LPS on basal and stimulated ECL cell histamine release was then measured. As in the previous experiments, incubation with gastrin significantly ($p < 0.05$) stimulated histamine levels, with stimulation higher at 10^{-8} M (1.36 fold) than at 10^{-10} M (1.24 fold). *H. pylori* LPS stimulated basal histamine secretion maximally at 10 ng/ml (1.69 ± 0.25 vs. basal, $p = 0.05$) (Figure 9.2-left panel), had no significant effect on maximal (10^{-8} M) gastrin-stimulated release, but significantly increased sub-maximal (10^{-10} M) gastrin-stimulated release with a maximal concentration of 100 ng/ml (1.48 ± 0.08, $p = 0.05$ vs. gastrin alone) (Figure 9.2-right panel).

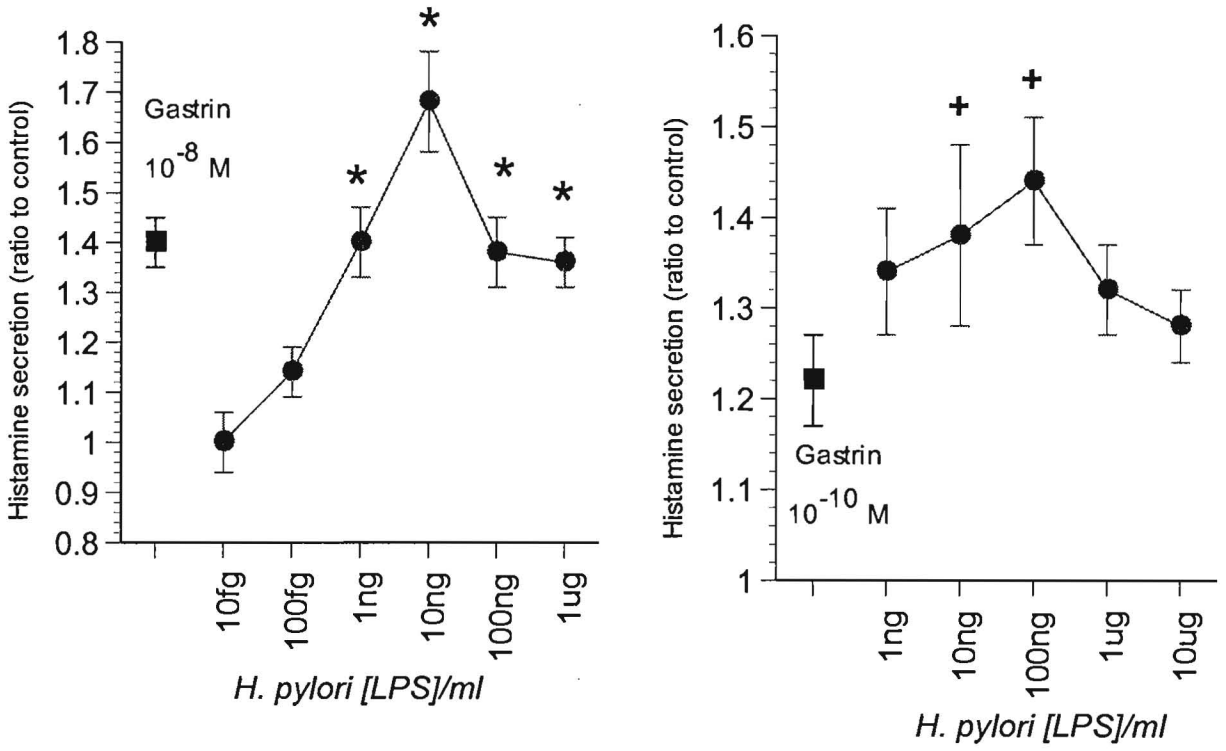


Figure 9.2 (left panel) Effect of *H. pylori* LPS on basal secretion. ■ Gastrin 10^{-8} M, ● LPS alone. $^{*}p = 0.05$ (n = 5).

Figure 9.2 (right panel) Effect of *H. pylori* LPS on sub-maximal gastrin-stimulated release. ■ Gastrin 10^{-10} M, ● LPS + gastrin (10^{-10} M). $^{+}p = 0.05$ (n = 4).

9.3.3 Lack of effect of the Gastrin/CCK$_B$ receptor antagonist, L365,260, on H. pylori LPS-stimulated histamine secretion

The major stimulatory mechanism for ECL cell histamine release is via activation of the gastrin receptor [22]. To determine whether *H. pylori* LPS stimulated histamine release by interaction with this receptor, the potential inhibitory effect of the specific receptor antagonist, L365,260, on *H. pylori* LPS-stimulated secretion was evaluated. Basal levels were 3.2 ± 0.18 nmoles/10^3 cells and as expected, both gastrin (10^{-8} M) and *H. pylori* LPS (100 ng/ml) stimulated histamine release (1.4 ± 0.1 and 1.6 ± 0.2 times basal respectively, $p = 0.01$). L365, 260 (10^{-8} M) significantly inhibited gastrin-stimulated histamine release (1.05 ± 0.04, $p < 0.05$) but at concentrations ranging from $10^{-12} - 10^{-6}$ M, had no effect on *H. pylori* LPS (100 ng/ml)-stimulated histamine secretion, making it unlikely that the stimulatory effect of *H. pylori* is mediated by the gastrin receptor (Figure 9.3).

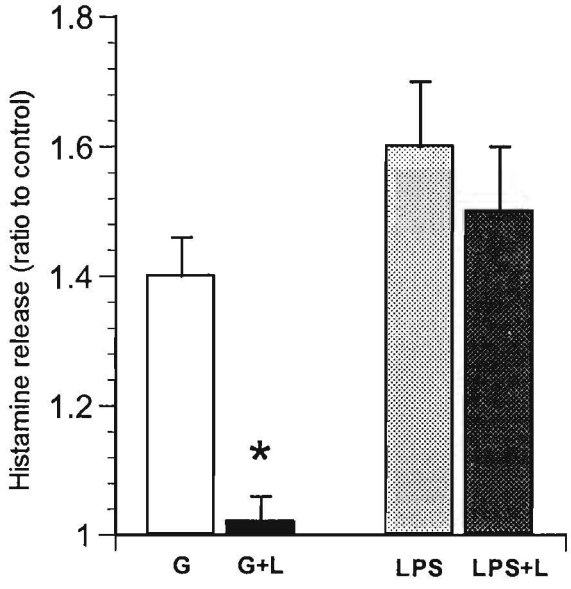


Figure 9.3 Effect of L365,260 (10^{-6} M) on gastrin or LPS-stimulated histamine release. G = gastrin (10^{-8} M), G+L = gastrin + L365,260 (10^{-6} M), LPS = *H. pylori* LPS (100 ng/ml), LPS + L = LPS and L365,260 (10^{-6} M). $^*p < 0.05$ (n = 3).

9.3.4 Inhibitory effect of somatostatin on H. pylori LPS-stimulated histamine secretion

Because a major inhibitory mechanism for basal and stimulated ECL cell histamine release is via activation of the somatostatin receptor [22], the effect of somatostatin on *H. pylori* LPS-stimulated histamine release was investigated. As in previous experiments, *H. pylori* LPS (100 ng/ml) significantly stimulated histamine release ($p = 0.02$). However, pre-treatment with somatostatin inhibited *H. pylori*-driven histamine secretion in a dose dependent manner with an estimated IC_{50} of approximately $5x10^{-13}$ M and maximum inhibitory concentration of 10^{-10} M ($p = 0.05$ vs. LPS alone) (Figure 9.4). These results are similar in magnitude to the inhibitory effect of somatostatin on gastrin stimulated histamine secretion [319]. Thus, the inhibitory effect of somatostatin appears to act on intracellular secretory pathways activated by both gastrin and *H. pylori* LPS.

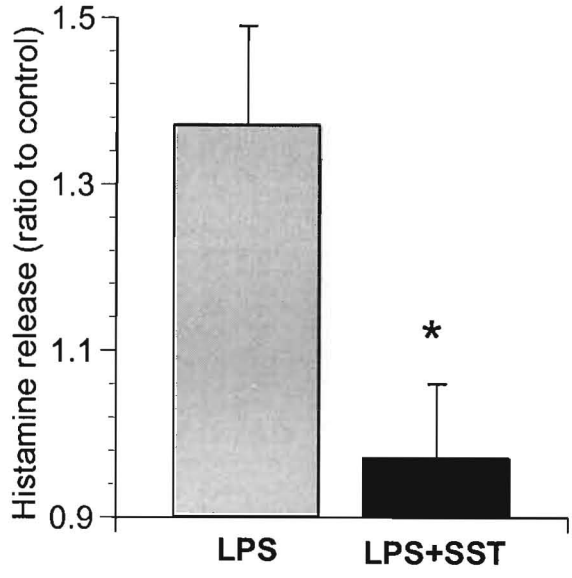


Figure 9.4 Effect of somatostatin (SST) on *H. pylori* LPS-stimulated histamine release. LPS = *H. pylori* LPS (100 ng/ml), LPS + SST = LPS + somatostatin (10^{-10} M). $^{*}p < 0.05$ ($n = 4$).

9.3.5 Lack of cytotoxic effect on ECL cells by H. pylori or E. coli LPS

The possibility that these LPS preparations altered histamine secretion via a cytotoxic effect was next evaluated. When assessed at high concentrations (10 ug/ml), neither *H. pylori* nor *E. coli* LPS had a significant effect on cell viability, measured either by trypan blue exclusion assessed over 24 hr (Figure 9.5-left panel) or by LDH release at 1 hr (Figure 9.5-right panel).

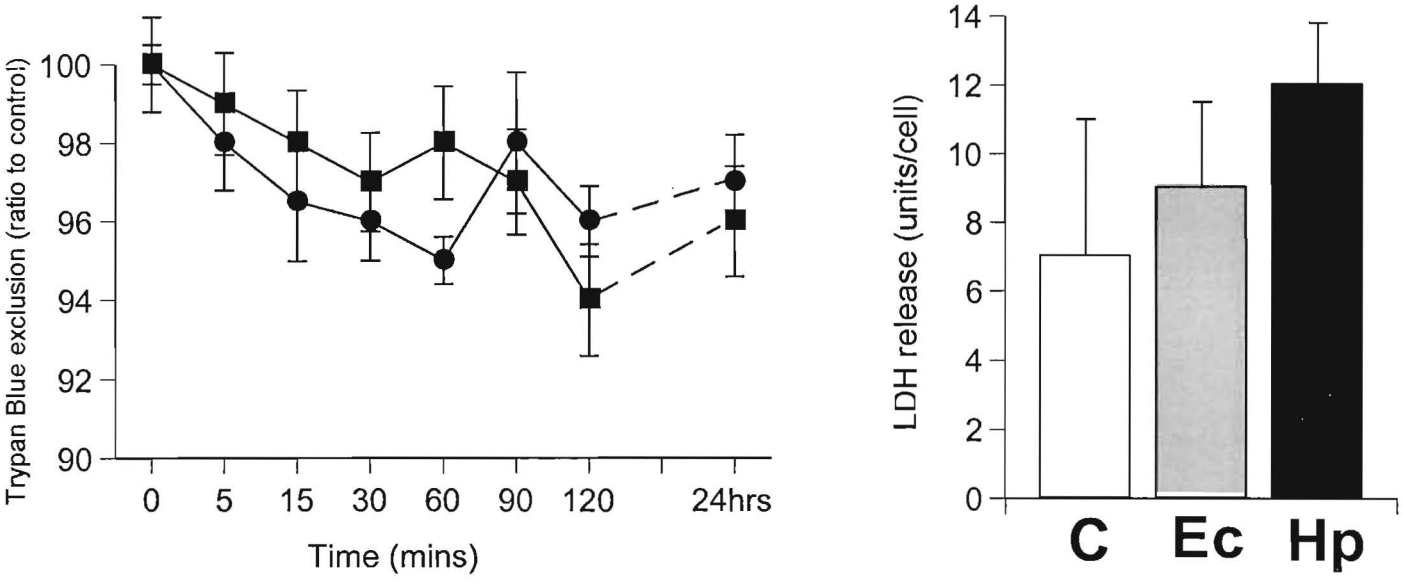


Figure 9.5 (left panel) Effect of LPS on ECL cell Trypan blue exclusion. ● *E. coli* LPS, ■ *H. pylori* LPS. (n = 3).

Figure 9.5 (right panel) Effect of LPS on LDH release. C = control (no LPS), Ec = *E. coli* LPS, Hp = *H. pylori* LPS. (n = 3).

9.3.6 Identification of CD14 message in ECL cells

The presence of CD14 messenger RNA in ECL cells was next identified. RT-PCR revealed the presence of a band of 430 bp in rat spleen (positive control). A similar band was obtained from rat ECL cell cDNA, demonstrating the presence of message for the LPS receptor on the ECL cell (Figure 9.6).

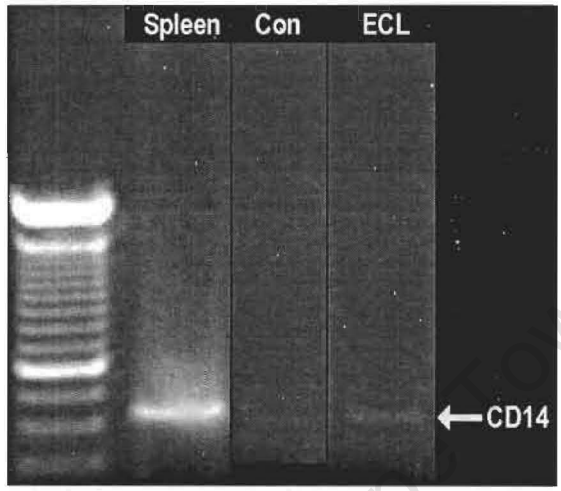


Figure 9.6 RT-PCR amplification of CD14. mRNA was prepared from rat spleen (positive control) and rat ECL cells. A blank control (Con) was also performed to exclude non-specific amplification. After reverse transcription, cDNA was quantified and CD14 message amplified. The expected priming sequence (432 bp) was detected in both samples. The gel is representative of 3 separate experiments.

9.3.7 Effect of E. coli and H. pylori LPS on DNA synthesis

The effect of *E. coli* LPS on basal and stimulated ECL cell DNA synthesis was initially evaluated. *E. coli* LPS significantly increased basal synthesis only at 10 ug/ml ($p < 0.01$), but did not substantially alter gastrin-stimulated DNA synthesis (Figure 9.7).

In parallel studies, *H. pylori* LPS alone (10 fg/ml – 10 ug/ml) had no effect, but augmented gastrin (10^{-8} M) driven DNA synthesis maximally at 100 ng/ml (2.5 ± 0.17 times the basal, ($p = 0.03$) (Figure 9.7). The maximal stimulatory concentration of LPS is the same both for gastrin-stimulated DNA synthesis and histamine release, suggesting common modifications at an intracellular level for these responses.

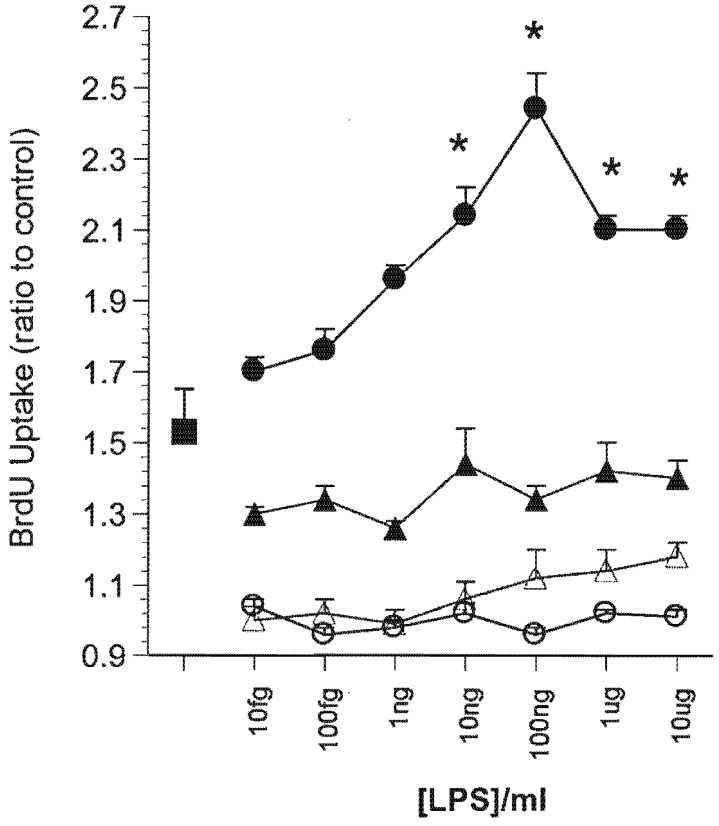


Figure 9.7 Effect of LPS on DNA synthesis. ■ Gastrin (10^{-8} M), Δ *E. coli* LPS, O *H. pylori* LPS, ▲ *E. coli* LPS + gastrin (10^{-8} M), ● *H. pylori* LPS + gastrin (10^{-8} M). $^{*}p < 0.05$ ($n = 5$).

9.4 Discussion

The results of the present study demonstrate that LPS alters both basal and gastrin-stimulated histamine secretion and that this effect may be bacterial species-specific. Of interest is that the combination of LPS and submaximal doses of gastrin resulted in an increased but not completely additive release of histamine from ECL cells. It is probable that this combination alters histamine release via modifications at a second messenger level.

The mechanism by which *H. pylori* LPS potentially mediates histamine secretion was thereafter investigated. Since co-incubation with L365,260 did not alter LPS-induced histamine secretion, it appears unlikely that *H. pylori* LPS alters histamine secretion via activation of the gastrin receptor. In addition, the stimulatory effect of LPS both on basal and gastrin-stimulated histamine release, which is in contrast to the autoinhibitory effect of the H_3 receptor [322] suggests that it is unlikely that LPS interacts with this receptor *in vitro*. In previously reported investigations it has been shown that somatostatin inhibits ECL cell histamine secretion with an IC_{50} of 10^{-13} M [319]. In this study it was noted that somatostatin inhibited *H. pylori* LPS induced histamine secretion in a dose dependent manner, suggesting that LPS-mediated histamine release is amenable to SST receptor induced modifications in second messenger pathways. The probability exists that *H. pylori* LPS could block the interaction between somatostatin and its receptor *in vitro* [323], and this study's observations could thus be explained by the presence of a contaminating population of D-cells in the ECL cell preparation. However, this appears to be unlikely since the percentage of such cells is low, typically less than 1%. In addition significant levels of the peptide in the cell cultures were not detected. Since the subtype 2 SST receptor is predominant on ECL cells [324,325], it is likely that the inhibition of histamine induced secretion occurs via this receptor subtype. Given that neither *E. coli* nor *H. pylori* LPS significantly altered ECL cell viability over a 24 hr period, it is probable that *H. pylori* LPS did not cause histamine release by physically damaging the cells.

The presence of both an LPS receptor (CD14) and the related intracellular pathways have been described previously in a number of immunological cell types [269]. Within the gut, this receptor has been identified only in the SW620 human colonic carcinoma cell line [326]. The presence of this receptor has not yet been investigated in the gastric mucosa. This study was able to identify CD14 message in the fundic ECL cell, thus confirming the presence of an LPS receptor and suggesting a mechanism by which *H. pylori* can directly affect this cell *in vivo*.

The observation that *H. pylori* LPS, while having no substantial effect on basal DNA synthesis resulted in a substantial augmentation of the trophic response of gastrin, resembles the physiology of fundic ECL cells exposed to elevated gastrin levels in association with elevated pH and fundic colonization of *H. pylori*. ECL cells are exquisitely sensitive to the trophic action of gastrin and undergo hyperplasia in a variety of hypergastrinemic conditions [22]. Experimental long-term (> 16 week) administration of the gastric H_2-receptor antagonist, loxtidine, prolonged acid inhibition, resulting in a secondary hypergastrinemia in rodents, *Praomys natalensis* (mastomys) [327]. The resulting hyperplastic proliferation of the oxyntic ECL cells generates gastric carcinoid tumors within four months [318,328]. In humans, ECL cell hyperplasia is generally reversible even after prolonged administration of proton pump inhibitors [22]. A marked increase in peripheral blood histamine levels and an increased urinary histamine excretion are also associated with ECL cell proliferation and histamine itself may function as a mitogen [329,330]. In experimental studies, the H_1 receptor subtype is expressed on the ECL cell and stimulates DNA synthesis [331].

H. pylori is classified as a gastric carcinogen (cf. Chapter 3) but a direct effect in the pathogenesis of ECL cell proliferation is not known. Several observations, however, implicate the bacterium in this process. Micronodular pseudohyperplasia probably represents a passive process of endocrine cell clustering and is frequently observed in foci of gland atrophy that may occur in *H. pylori*-gastritis [124,126,332]. The phenomenon of endocrine cell clustering appears to be restricted to patients with *H. pylori* colonization but has not been demonstrated to progress beyond micronodular hyperplasia, which only occasionally has been of the pre-dysplastic adenomatoid type [126,127,332]. It may be noteworthy, however, that such cell clustering is more marked in fundal than in antral gastritis [102], and may reflect a direct mitogenic effect of the bacterium on endocrine cells. Long term usage of proton pump inhibitory (PPI) therapy may result both in a migration of the organism from the antrum to the fundus [333] as well as an accentuation of the gastritis and atrophy in patients [334] although this may be controversial. The combination of long term treatment with PPI's (5 years) and *H. pylori* infection has been demonstrated to be associated with an increase in ECL cell hyperplasia in one study [335]. It has thus been proposed that the organism might be an important risk factor for ECL cell proliferation in certain clinical settings. The mechanisms by which this occurs is currently open to conjecture. However, the production of autoantibodies against Lewis x antigens observed in patients infected with the organism [262,264] suggests that the Lewis x glycoconjugate (which is mimicked by LPS of *Helicobacter*

pylori) is able to enter the gastric mucosa. In addition, it is likely that LPS may enter via mucosal micro-lesions associated with the infection. It is thus feasible that local concentrations adequate to stimulate the ECL cell occur, especially given the propensity of the organism to autolyze during colonization [236]. In addition, although this has not yet been documented, the possibility exists that LPS may be present in the plasma of infected individuals as are many endotoxins. This could provide an alternative mechanism for the stimulation of the ECL cell *in vivo*.

9.5 Conclusion

A model endocrine system examining the effects of LPS on a biologically relevant gastric cell has been demonstrated. This study has revealed both a proliferative and a secretory effect of a toxigenic *H. pylori* LPS on the naïve rat ECL cell. The presence of CD14 suggests a mechanism by which this may occur. It seems plausible that similar effects may occur *in vivo*, where direct *H. pylori* stimulation of ECL cells may result in altered acid secretion and gastric mucosal proliferation. In addition, it is possible that a similar effect on other cells of the oxyntic mucosa could be relevant to the carcinogenicity of *H. pylori*.

Chapter 10

Analysis of the effects of LPS on transformed ECL cells

10.1 Introduction

Infection by *H. pylori* may evolve into atrophic gastritis, with increased mucosal proliferation and the predisposition to neoplastic transformation (reviewed more fully in Chapter 3) [336,337]. Although atrophy and destruction of mucosal glands, balanced by a "compensatory" gastric proliferative response has been hypothesized to result in mucosal transformation [107], the mechanisms by which this may occur are currently open to conjecture.

Several studies have documented that polyamine homeostasis, a tightly regulated, ubiquitous eukaryotic cellular phenomenon responsible for mediating growth responses to most agents stimulating cell growth including gastrin and growth factors [338], is important in the development of gastrointestinal neoplasia. The activity of the rate-limiting enzyme, ornithine decarboxylase (ODC) is elevated in gastric cancer [339,340]. ODC activity is also increased in patients infected with *H. pylori* and eradication of the organism has been demonstrated to result in a decreased ODC activity, and by inference, the potential for a decreased risk of gastric cancer [341-343].

A role for *H. pylori* LPS in mediating both histamine release and DNA synthesis in naive rat ECL cells *in vitro* has been identified (cf. Chapter 9), while a role for polyamines in the regulation of gastrin-mediated ECL cell DNA synthesis has been demonstrated [344]. One may postulate that *H. pylori* directly affects proliferation in tumor ECL cells via LPS activation of DNA synthesis and activation of polyamine biosynthesis (ODC activity). Furthermore, based on the observation that CD14 is a putative receptor for LPS, the possibility that such a phenomenon could be mediated via interaction with the CD14 receptor was therefore examined.

10.2 Materials and Methods

10.2.1 Materials

All materials, including *E. coli* LPS (serotype 026:B6), were obtained commercially from Sigma Chemical Co. (St. Louis, Missouri, USA) except where otherwise indicated. The cytotoxic ($VacA^+/CagA^+$) *H. pylori* LPS (strain 84-183) was a kind gift from M.J. Blaser and G.I. Perez-Perez (Division of Infectious Diseases, Vanderbilt University, Tennessee, USA). Loxtidine, an irreversible H_2 receptor blocker, was a kind gift of Glaxo UK, (Ware, England)

and the irreversible inhibitor of ODC activity, difluoromethyl-ornithine (DFMO), was a kind gift from Dr. E. Bohme, Marrion Merrell Dow Research Institute, (Cincinnati, Ohio, USA). Human gastrin-17 and rat TGFα was obtained from Research Plus Inc. (Bayonne, New Jersey, USA), BCA protein assay reagent from Pierce (Rockford, Illinois, USA), ^{14}C-ornithine from New England Nuclear (Boston, Massachusetts, USA) (43.8 mCi/mmol), 5-bromo-2-deoxyuridine (BrdU) proliferation kit from Amersham Corp (Arlington Heights, Illinois, USA), collagen-I coated 96 well plates from Becton Dickinson (Bedford, Massachusetts, USA), Nycodenz from Accurate Chemical and Scientific Corp (Westbury, New York, USA), and Pronase E from Boehringer Mannheim (Indianapolis, Indiana, USA). The mouse monoclonal antibody, M-M42, against CD14, was obtained from Novocastra Laboratories Ltd., Newcastle upon Tyne, UK.

10.2.2 Animals

All animals for these studies were bred from the mastomys colony (ModYale) maintained at the West Haven Department of Veterans Affairs Medical Center, West Haven, Connecticut, USA. The animals were maintained in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. Animals with equal sex distribution were assigned to receive loxtidine (1 mg/kg/day) delivered in drinking water (2 g/liter) for 16 weeks. All animals had free access to rodent chow (Purina, St. Louis, Missouri, USA). Hypergastrinemia was confirmed by gastrin RIA [345].

10.2.3 Isolation of ECL cells

Mastomys tumor ECL cells were prepared as previously described [345]. From each loxtidine-treated mastomys, macroscopic carcinoids, mainly free of other gastric mucosal cells, were isolated and minced by hand, and individual cells released by alternate pronase (3 mg/ml) and collagenase (3 mg/ml) digestion. Cells were either immediately snap frozen and stored at -70 °C for mRNA extraction, used within an hr. in ODC assays or cultured in culture medium (DMEM:F12 and 2% BSA, pH 7.4) at a final concentration of 10^5 cells/ml, 100 µl/well for DNA studies.

10.2.4 Experimental design

An isolated, purified preparation of mastomys tumor ECL cells was used in all experiments [345].

10.2.4.1 Polyamine biosynthesis in tumor ECL cells: To evaluate an effect on DNA synthesis, cells were incubated with either TGFα, putrescine, spermidine or spermine alone (10^{-12} - 10^{-6} M), or with a combination of each polyamine (10^{-12} - 10^{-6} M) and a maximal concentration of

TGFα (10^{-9} M) for 24 hr. To examine whether the effect of polyamines and TGFα was mediated via ODC, the effect of DFMO on TGFα (10^{-9} M) stimulated DNA synthesis was next evaluated. Thereafter the effect of TGFα (10^{-13} - 10^{-9} M) on the activity of this enzyme was examined using a modified radiometric technique [346]. In addition, in order to examine whether gastrin had an effect on tumor cells the ability of this agent (10^{-11} - 10^{-7} M) to alter ODC activity was thereafter evaluated.

10.2.4.2 LPS effects on tumor ECL cells: To evaluate an effect on DNA synthesis, cells were incubated with either a maximal concentration of TGFα (10^{-9} M) (positive control) or with *H. pylori* LPS alone (10 fg/ml – 1 ug/ml) for 24 hr LPS from *E. coli* (1 ug/ml) was used as a non-gastric control. To examine whether the proliferative effect of *H. pylori* LPS was mediated via polyamine biosynthesis and ODC, we next evaluated the effect of the irreversible inhibitor of ODC, DFMO (10^{-11} - 10^{-8} M), on LPS (10 ng/ml) stimulated DNA synthesis. To further examine whether the effect of *H. pylori* LPS was via activation of this pathway, we evaluated the effect of this agent alone (1 ng/ml – 1 ug/ml) on the activity of ODC. TGFα (10^{-9} M) and *E. coli* LPS (1 ug/ml) were used as controls. The presence of the CD14 receptor was identified by RT-PCR from cDNA derived from the tumor cells. In order to confirm whether the CD14 receptor played a role in mediating LPS stimulated ECL cell DNA synthesis, we assessed the effect of pre-incubation for 5 min with the specific monoclonal antibody against CD14, M-M42, (1:250 - 1:50) on *H. pylori* LPS (10 ng/ml) stimulated BrdU uptake.

10.2.5 Cell proliferation assay by BrdU incorporation

After 24 hr in culture (96-well collagen-I coated plate, 10^4 cells/well) at 37°C (5% CO_2), medium was aspirated and replaced with culture medium containing BrdU (pre-diluted labeling reagent (1:200) - Amersham) and the indicated agent (TGFα or LPS) as previously described [345]. After incubation for an additional 24 hr, the medium was removed and the cells fixed at 4°C in 90% ethanol/5% acetic acid/5% water, then washed in 0.1% Tween-20 in phosphate buffered saline (PBS, pH 7.4). The wells were incubated with anti-BrdU antibody with nuclease (1:400 - Amersham) for 1 hr at room temperature, and following 3 washes, incubated with rabbit anti-mouse IgG conjugated to horseradish peroxidase (1:800 - Amersham) for 30 min at 20°C. After a further 3 washes, wells were incubated with substrate (30 mg O-phenylenediamine dihydrochloride in citrate-phosphate buffer, pH 4.1), and optical density determined at 405 nm by spectrophotometry (Biorad Microplate reader model 450,

Winooski, Vermont, USA). Results are expressed as OD units$_{(405\ nm)}$ and final results are expressed as ratio of mean ± SEM to control.

10.2.6 Evaluation of ODC activity

Freshly isolated cells (10^5/100 µl culture medium) were incubated with either TGFα (10^{-9} M) or *H. pylori* LPS (1 ng/ml – 1 ug/ml) for 1 hr at 37°C. *E. coli* LPS was used as a non-gastric control and unstimulated cells provided values for basal ODC activity. ODC activity was then assayed as previously described [344]. Briefly, after stimulation, cells were homogenized in ice cold Tris-HCl buffer (15 mM Tris-HCl, 0.1 mM EDTA, 2.25 mM dithiothreitol (DTT), pH 7.5), centrifuged and an aliquot of the cytosolic extract removed and later assayed for protein content using the bicinchoninic acid (BCA) method. A second aliquot of the cytosolic extract was immediately incubated in the reaction buffer containing radiolabeled ^{14}C-ornithine (200 nCi), for 1 hr at 37°C, the reaction terminated by addition of HCl (37%) and the liberated product extracted in Betablend over 30 min at 37°C, and counted. Final results (dpm/µg protein/1 hr) were expressed as ratio to control.

10.2.7 RT-PCR for CD14

The FastTrack mRNA isolation kit (Invitrogen, San Diego, CA) was utilized to isolate mRNA from ECL cells. SuperScript preamplification system (Gibco BRL, Gaithersburg, Maryland, USA) was utilized to prepare first strand cDNA. Specific DNA amplification for CD14 was carried out with the following combinations of primers derived from the CD14 nucleotide sequence [268]: sense: 5'-CTTGAACCTCCGCAACGTGTC and antisense: 5'-CCCAGTGAAAGACAGATTGA. PCR using 0.7 µg cDNA template was carried out with Taq DNA polymerase through 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 45°C) and extension (1 min at 72°C) on a thermal cycler from MJ Research (Watertown, Massachusetts, USA). Products were electrophoretically separated and visualized using UV.

10.2.8 Evaluation of the effect of M-M42 on DNA synthesis

M-M42 at a final concentration of 1:250 to 1:50 was added 5 min prior to adding *H. pylori* LPS (1 ng/ml) to cells which were then incubated overnight. Alterations in BrdU accumulation were measured as described above.

10.2.9 Evaluation of LPS cytotoxicity

To assess non-specific damage to the ECL cells, cell viability was assessed by Trypan blue exclusion as previously described [347]. Briefly, cells were incubated with either *H. pylori* or *E. coli* LPS (both 10 ug/ml) in PBS for 0, 1, 2 and 24 hr. Alterations in viability were compared to control levels (0 hr) and final results were expressed as percentage of controls.

10.2.10 Statistical evaluation

Results are expressed as mean ± SE. "n" indicates the number of ECL cell preparations. Statistical analysis was performed using the two-tailed Student's t-test for paired values as appropriate and p-values < 0.05 were considered significant.

10.3 Results

10.3.1 Polyamine biosynthesis in tumor ECL cells

a) Effect of polyamines on DNA synthesis

The effect of putrescine, spermidine and spermine on basal and stimulated tumor ECL cell DNA synthesis was initially measured. All of the polyamines significantly increased (~30%) basal DNA synthesis over the 24 hr experimental period (Figure 10.1-left panel). The estimated EC_{50} values were 3 x 10^{-12} M for putrescine and 2 x 10^{-10} M for spermidine and spermine, respectively. In addition, all of the polyamines significantly augmented (~40%) the stimulatory effect of TGFα (10^{-9} M) (Figure 10.1-right panel). The estimated EC_{50} values were 2 x 10^{-11} M for putrescine, 2 x 10^{-10} M for spermidine and 3 x 10^{-10} M for spermine, respectively.

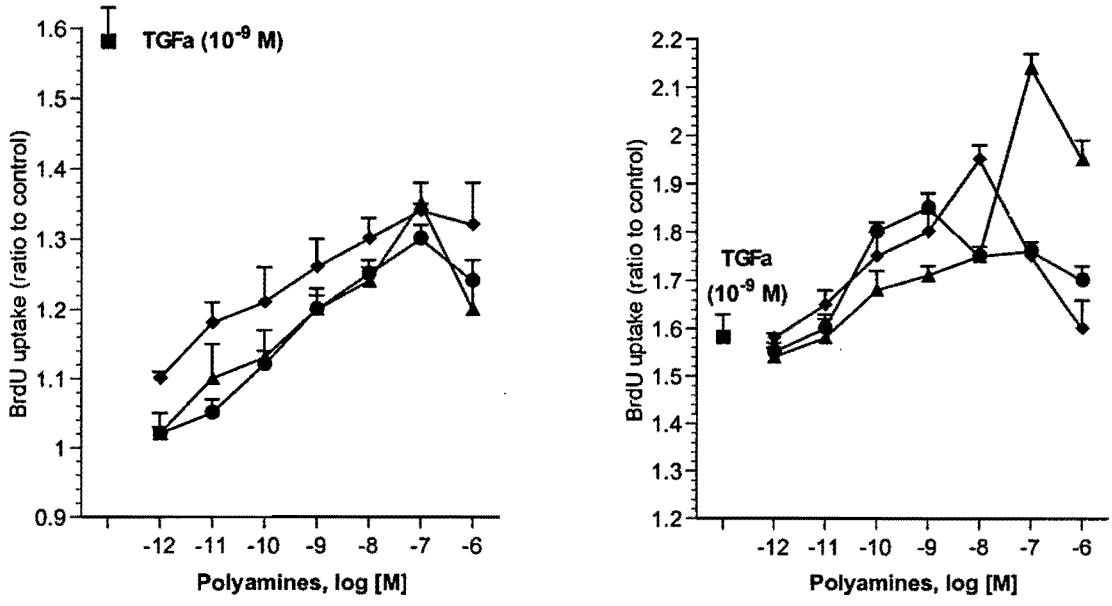


Figure 10.1 (left panel) Effect of polyamines on unstimulated ECL cell tumor DNA synthesis. ■ TGFα (10^{-9} M), ◆ putrescine, ● spermidine, ▲ spermine. (n = 6).

Figure 10.1 (right panel) Effect of polyamines on TGFα-stimulated ECL cell tumor DNA synthesis. ■ TGFα (10^{-9} M), ◆ putrescine + TGFα (10^{-9} M), ● spermidine + TGFα (10^{-9} M), ▲ spermine + TGFα (10^{-9} M). (n = 5).

b) Effect of DFMO on DNA synthesis

The effect of DFMO, on basal and stimulated tumor ECL cell DNA synthesis was next measured. As in previous experiments, incubation with TGFα (10^{-9} M) significantly (1.58 ± 0.02, $p < 0.01$) stimulated DNA synthesis (Figure 10.2). DFMO alone did not have a substantial inhibitory effect on basal DNA synthesis (Figure 10.2) but pre-treatment of the cells for 30 min with this agent dose dependently inhibited TGFα (10^{-9} M) stimulated DNA synthesis with an IC_{50} of approximately 3 x 10^{-12} M and a maximal inhibition of ~90% at 10^{-9} M (1.07 ± 0.15, $p < 0.05$ vs. TGFα).

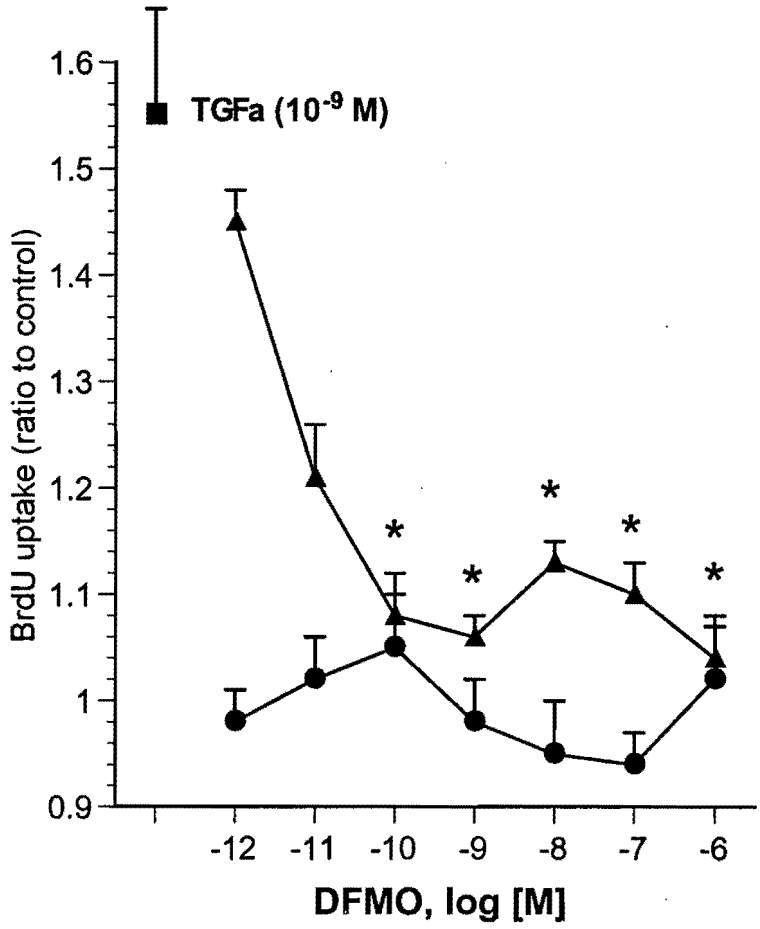


Figure 10.2 Effects of DFMO on DNA synthesis. ■ TGFα (10^{-9} M), ● DFMO alone, ▲ DFMO + TGFα (10^{-9} M). *$p < 0.05$ vs. TGFα alone (n = 5).

c) TGFα stimulation of DNA synthesis and ODC activity

The effect of TGFα on DNA synthesis and ODC activity in ECL tumor cells was then measured. TGFα stimulated DNA synthesis over 24 hr with an estimated EC_{50} of 5×10^{-11} M and a maximal concentration of 10^{-10} M (1.92 ± 0.14, $p < 0.01$) (Figure 10.3).

TGFα stimulated ODC activity with an estimated EC_{50} of 3×10^{-12} M and a maximal concentration of 10^{-11} M (1.5 ± 0.08, $p < 0.01$). Pre-treatment of the cells for 30 min with DFMO (10^{-8} M) reversed the stimulatory effect of TGFα (10^{-11} M) on ODC activity (1.17 ± 0.07, $p < 0.05$ vs. TGFα alone). Gastrin had no effect on tumor ECL cell ODC activity. This is consistent with our previous observation that the effect of gastrin on tumor cell DNA synthesis is negligible [345].

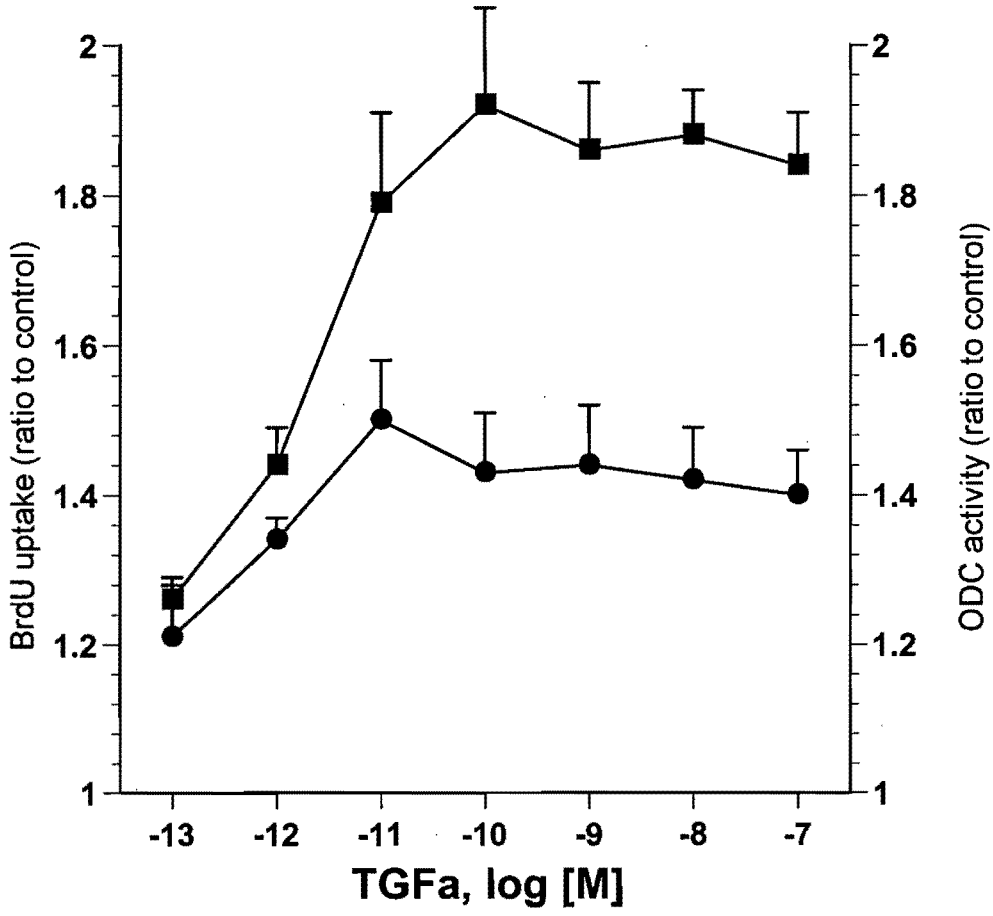


Figure 10.3 Effects of TGFα on BrdU uptake (■) and ODC activity (●) (n = 4).

10.3.2 Effect of H. pylori LPS on DNA synthesis

The effect of LPS on basal DNA synthesis in mastomys tumor cells was then measured. TGFα (10^{-9} M) stimulated DNA synthesis as expected (1.6 ± 0.08, $p < 0.05$ vs. control) (Figure 10.4). LPS from *E. coli* (1 ug/ml) had no significant effect on BrdU uptake over the 24 hr experimental period (1.28 ± 0.14, p = NS vs. control). In contrast, LPS from *H. pylori* significantly stimulated DNA synthesis in mastomys tumor cells with an estimated EC_{50} of 50 fg/ml, and a maximal effect of 2.2 fold.

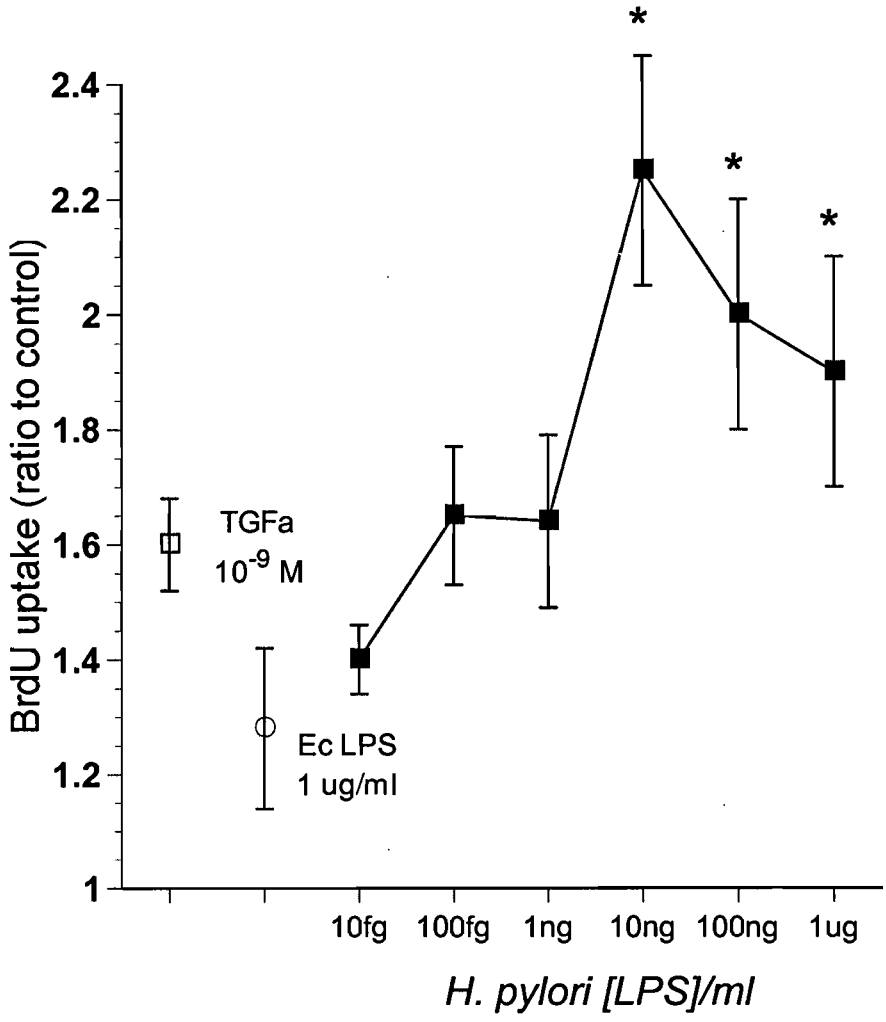


Figure 10.4 Effect of LPS on DNA synthesis. □ TGFα 10^{-9} M, O *E. coli* LPS (1 ug/ml), ■ *H. pylori* LPS. *$p < 0.01$ vs. control (n = 4).

10.3.3 Effect of DFMO on H. pylori LPS stimulated DNA synthesis

The effect of DFMO on *H. pylori* LPS stimulated DNA synthesis was next evaluated. Incubation with LPS (10 ng/ml) significantly (2.08 ± 0.2, $p < 0.01$ vs. control) stimulated DNA synthesis (Figure 10.5). Whilst DFMO alone (10^{-11} – 10^{-8} M) had no significant inhibitory effect on basal DNA synthesis, pre-treatment of the cells for 30 min with this agent dose dependently inhibited LPS (10 ng/ml) stimulated DNA synthesis with an approximate IC_{50} of 2×10^{-11} M and a maximal inhibition of ~75% at 10^{-10} M (1.25 ± 0.18, $p < 0.05$ vs. LPS alone).

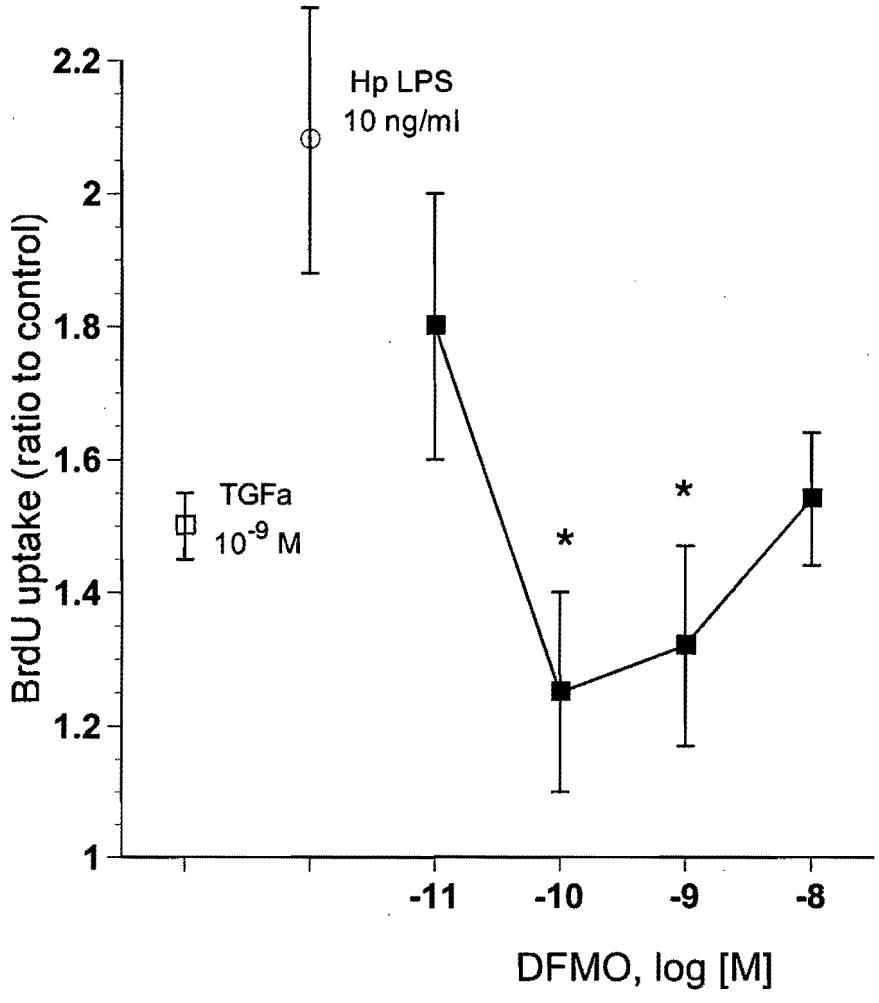


Figure 10.5 Effect of DFMO on DNA synthesis. □ TGFα (10^{-9} M), O *H. pylori* LPS (10 ng/ml), ■ DFMO + *H. pylori* LPS (10 ng/ml). *$p < 0.05$ (n = 3).

10.3.4 H. pylori LPS stimulation of ODC activity

The effect of LPS on ODC activity in freshly isolated cells was thereafter examined. TGFα (10^{-9} M) significantly stimulated ODC activity (measured by $^{14}CO_2$ release from ^{14}C-ornithine) (1.47 ± 0.04, $p < 0.01$ vs. control) (Figure 10.6). While LPS from *E. coli* (10 ug/ml) had no effect, *H. pylori* LPS significantly augmented ODC activity with an estimated half-maximal concentration of 1 ng/ml and a maximal effect of 1.4 fold ($p = 0.05$ vs. control). The specificity of this response was determined when pre-incubation of the cells with DFMO (10^{-9} M) for 30 min reversed the stimulatory effect of *H. pylori* LPS (10 ng/ml) (1.02 ± 0.06 vs. 1.3 ± 0.03, $p = 0.05$ vs. LPS alone).

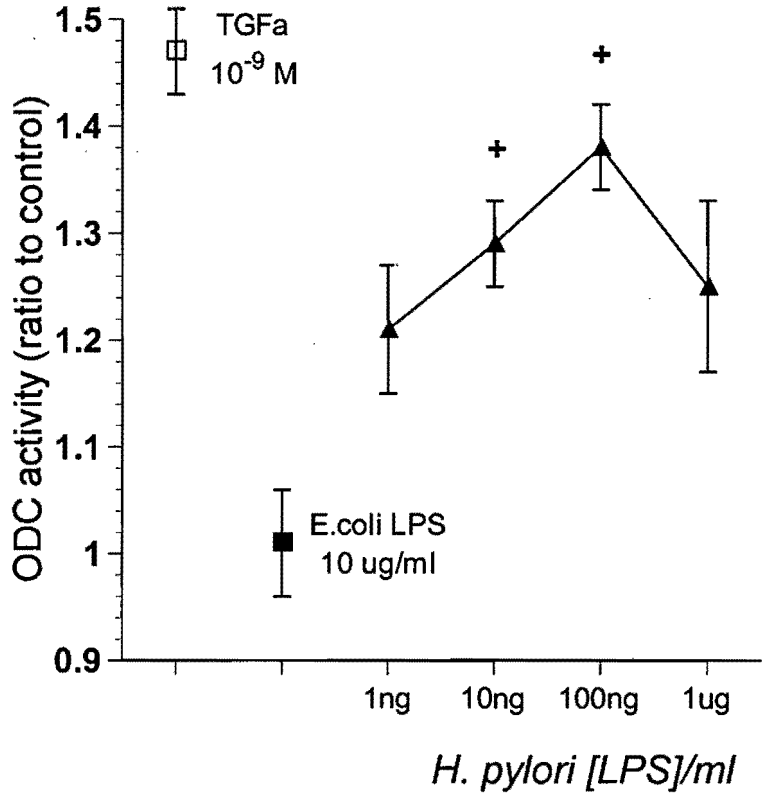


Figure 10.6 Stimulation of ODC activity by *H. pylori* LPS. □ TGFα 10^{-9} M, ■ *E. coli* LPS (10 ug/ml), ▲ *H. pylori* LPS. $^{+}p = 0.05$ (n = 4).

10.3.5 Identification of the CD14 receptor

The identification of the presence of CD14 messenger RNA in tumor ECL cells was next attempted. RT-PCR revealed the presence of a band of 430 base pairs in mastomys spleen (positive control). A similar band was obtained from mastomys tumor ECL cell cDNA, demonstrating the presence of the message for the LPS receptor on the ECL cell (Figure 10.7).

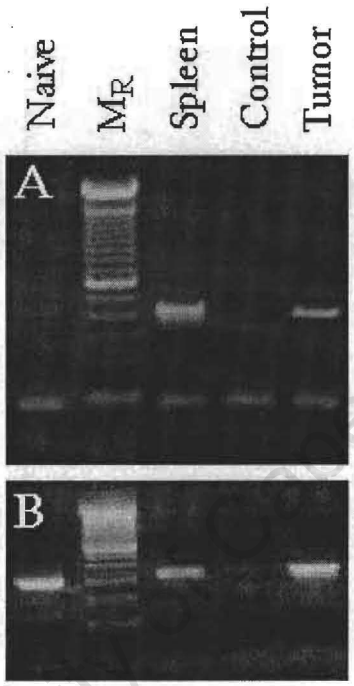


Figure 10.7 Identification of CD14 in mastomys *A*: Following reverse transcription, a band of 430 base pairs was identified by PCR from naïve ECL cells, spleen (positive control), and from tumor cells. A blank control was also performed to exclude non-specific amplification. M_R = Molecular weight marker. *B*: The amount of cDNA from each sample was standardized by PCR amplification of the housekeeping gene, glyceraldehyde 3'-phosphate dehydrogenase (GAPDH). The lanes are the same as in A. The gel is representative of 3 separate experiments.

10.3.6 Effect of M-M42 on LPS mediated DNA synthesis

Thereafter, the possibility that CD14 receptor mediated the stimulatory effects of *H. pylori* LPS in the tumor ECL cell was investigated. As in previous experiments, incubation with *H. pylori* LPS (10 ng/ml) resulted in a significant stimulation of DNA synthesis (2.08 ± 0.14, $p < 0.01$) (Figure 10.8). Pre-incubation with the monoclonal antibody against CD14, M-M42, for 5 min resulted in a dose dependent reversal of *H. pylori* LPS stimulated DNA synthesis with a maximal effect at a final concentration of 1:50 (1.18 ± 0.1, $p < 0.01$ vs. LPS alone). Pre-incubation of cells with M-M42 alone had no effect on basal DNA synthesis.

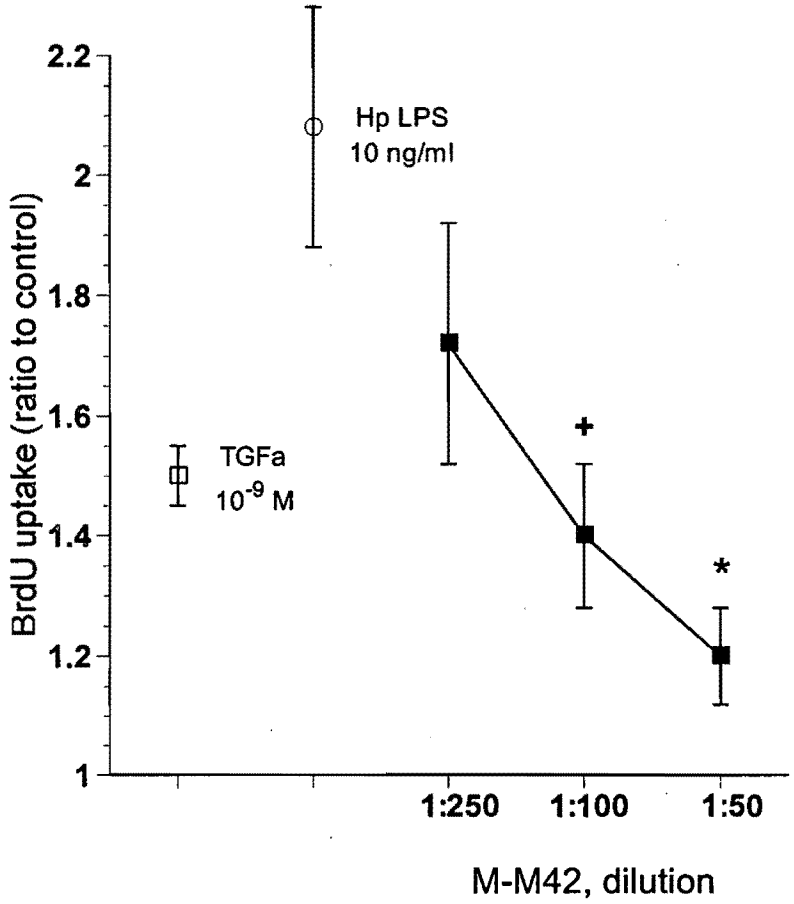


Figure 10.8 Inhibitory effect of M-M42 on *H. pylori* LPS mediated DNA synthesis. □ TGFα 10^{-9} M, O *H. pylori* LPS (10 ng/ml), ■ M-M42 + *H. pylori* LPS (10 ng/ml). $^{+}p < 0.05$, $^{*}p < 0.01$ (n = 3).

10.3.7 Cytotoxicity of LPS

When assessed at high concentrations, neither *H. pylori* nor *E. coli* LPS (10 ug/ml) had any significant effect on tumor ECL cell viability measured by uptake of Trypan blue over the 24 hr period (Figure 10.9).

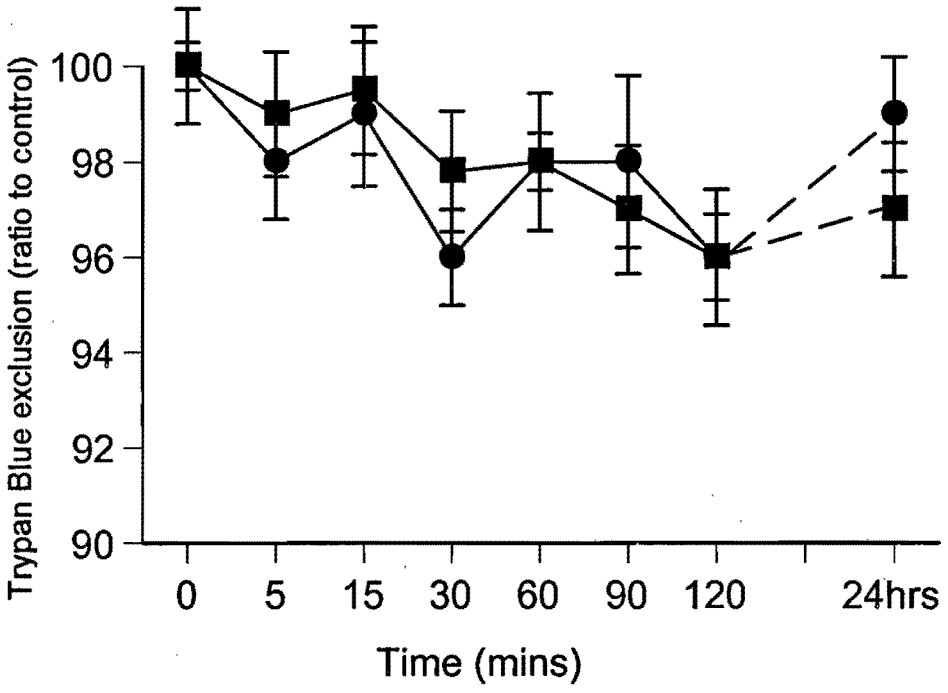


Figure 10.9 Effect of *E. coli* LPS (●) and *H. pylori* LPS (■) on ECL cell Trypan blue exclusion. (n = 3).

10.4 Discussion

The gastric endocrine ECL cell plays a critical role in the regulation of gastric exocrine function. Of particular pathological significance is the self-replicative nature of ECL cells under long term hypergastrinemia in mastomys and rats [345]. Gastrin as a physiological hormone promotes cell proliferation, and sustained hypergastrinemia may culminate in ECL cell hyperplasia and neoplasia [318,345]. The loss of gastrin "control" is associated with the involvement of alternative growth regulatory mechanisms such as a TGFα autocrine pathway [345]. Although gastrin and TGFα activate different post receptor second messenger systems, it is not known whether they share a common downstream intracellular denominator that is involved in activation of the DNA synthesis process.

Polyamines are especially evident in rapidly proliferating cells and tissues such as developing embryos and tumors, which have higher putrescine and spermidine concentrations than non-growing tissues [338]. These positively charged amines alter DNA conformation and catalyze nucleosome condensation - effects considered to be of importance in establishing the conditions necessary for DNA synthesis [348,349]. In addition, increases in ODC activity and polyamine synthesis are evident in cells in the G1 phase of cell division cycle [338], further supporting the critical role for ODC activity in cell proliferation. Alterations in polyamine homeostasis (by the addition of DFMO which inhibits ODC activity) have previously been demonstrated to result in growth inhibition of a pancreatic neuroendocrine cell line (BON) [350]. Since addition of polyamines to neuroendocrine tumor ECL cells resulted in stimulation of basal DNA synthesis (approximately 30%) it seems probable that polyamines have a direct mitogenic effect on tumor ECL cells. Of particular interest was the observation that the mitogenic effect of TGFα was significantly augmented by all tested polyamines. This is consistent with previous observations that growth factors increase both polyamine uptake, as well as intracellular polyamine concentrations, and DNA synthesis [351-353]. Pre-incubation with DFMO completely inhibited TGFα-stimulated DNA synthesis. This suggests that the TGFα mediated pathway is largely driven via ODC activity and polyamine biosynthesis and may be a reflection that tumor cells in general are more dependent on polyamine biosynthesis than their normal counterparts. This observation is consistent with reports that in quiescent fibroblasts, fibroblast growth factor and platelet-derived growth factor all stimulate ODC activity through at least one pathway involving protein kinase C [354]. It was unexpected that DFMO had no effect over 24 hr on basal DNA synthesis. This may reflect the fact that ECL cells are well differentiated, and are not rapidly proliferating.

In addition, TGFα stimulated tumor ECL cell ODC activity with an EC_{50} of 10^{-13} M, a value consistent with that of the effect on tumor cell DNA synthesis (10^{-11} M). This supports the conclusion that the proliferative effect of TGFα is mediated almost exclusively by this pathway in ECL tumor cells. This proposal is further strengthened by the observation that DFMO completely inhibits TGFα stimulated DNA synthesis.

In this study, a specific proliferative effect for *H. pylori* LPS on tumor ECL cell DNA synthesis was demonstrated. This effect was potent (EC_{50} ~0.1 ng/ml), 2.2 fold over basal. The efficacy and potency of this effect is similar to that of the principal regulator of tumor ECL cell proliferation, TGFα (5 x 10^{-11} M and 1.9 fold, respectively) [344]. While *H. pylori* LPS had no effect on basal naive (rat) ECL cell DNA synthesis, it could augment gastrin-stimulated DNA synthesis (cf. Chapter 9). The potent effect of LPS on "unstimulated" tumor cell DNA synthesis could either be a result of a direct mitogenic effect, or could reflect the relatively increased number of mitogenic cells potentially present in a preparation of tumor cells.

It has previously been demonstrated that polyamine homeostasis is an important mechanism by which tumor cell proliferation is mediated [344]. Thus, the addition of the irreversible ODC enzyme inhibitor, DFMO, potently and dose dependently inhibited LPS stimulated DNA synthesis. The potency (2 x 10^{-11} M) and efficacy (~75% inhibition) was similar to that reported for inhibition of TGFα mediated DNA synthesis (3 x 10^{-12} M, and ~90%) [344]. Analysis of ODC activity demonstrated a significant stimulation by *H. pylori* LPS (EC_{50} 1 ng/ml, 1.4 fold vs. control). These values are somewhat similar to that of TGFα (10^{-11} M, 1.5 fold vs. control), and suggest that LPS mediates tumor cell proliferation almost exclusively via activation of the biosynthetic pathway, and confirms the relevance of this pathway to tumor ECL cell DNA synthesis.

CD14 is a myeloid differentiation antigen, and its expression has been correlated with various forms of malignant myeloid leukemias [355]. Within the gut, this receptor has been identified only in the SW620 human colonic carcinoma cell line [326] and in the naïve ECL cell (cf. Chapter 9). The additional identification of CD14 message in the transformed ECL cell and its protein (albeit indirectly), supports the presence of an LPS receptor on this neuroendocrine cell and provides an explanation for the mechanism by which *H. pylori* can directly affect ECL cells *in vivo*.

Studies analyzing the relationship between CD14, LPS and proliferation have concentrated on cell lines derived from the immune system. In these experiments, addition of

LPS to the monocytic Mono Mac 6 cell line resulted in an up regulation of CD14 expression (mRNA and protein) and retarded proliferation [356,357]. In contrast, addition of *H. pylori* LPS to a neuroendocrine cell system resulted in proliferation, via activation of the CD14 receptor. This apparent contradiction suggests that CD14 may have different functions in different cell systems. Similar findings have been noted for the inhibitory neuropeptide somatostatin, which, through its normal mechanism of decreasing intracellular cAMP, inhibits intestinal epithelial proliferation in cultured cells [358], and induces proliferation in human meningioma and pancreatic carcinoid cells [359,360]. Unraveling the CD14 mediated intracellular pathway in the ECL cell may answer this question.

10.5 Conclusion

The relationship of *H. pylori* to gastric neoplasia is clinically relevant and the ECL cell related interactions may be involved in this association. Products of *H. pylori* may either directly stimulate ECL cell proliferation or accentuate histamine secretion which itself may be mitogenic. In addition, the associated increased gastrin levels are linked to both ECL cell and fundic mucosal cell proliferation. Since the ECL cell itself produces growth factors such as TGFα [345], it is possible that an *H. pylori* "activated" mucosa may be exposed to multiple mechanisms predisposing to neoplasia. A direct mitogenic effect of *H. pylori* LPS on carcinoid ECL cells *in vitro* has also been demonstrated, as well as the probability that LPS can initiate this polyamine mediated effect by binding to the CD14 receptor. It is possible that *H. pylori* may influence mucosal function *in vivo* via its effects (CD14 activation) not only on exocrine cells but on cells of the neuroendocrine cell system of both the fundus and the antrum.

Chapter 11.

Analysis of the effects of LPS on transformed gastrointestinal cells

11.1 Introduction

A role for *H. pylori* LPS obtained from a toxigenic (84-183) isolate in modulating DNA synthesis in both the naïve and transformed neuroendocrine ECL cell has been demonstrated (cf. Chapters 9 & 10). This effect is potent (10 ng/ml), is mediated by CD14 and appears to be specific to *H. pylori*.

A number of studies have evaluated the effects of *H. pylori* or its products on the cell cycle (reviewed more fully in Chapter 3). An analysis of these studies suggest that *H. pylori* products (sonicates and water extracts) are less effective than *E. coli* at stimulating ^{3}H-thymidine uptake in the IEC-6 small intestinal epithelial cell line [18,19]. Co-culture studies with the gastric AGS cell line demonstrate that the *H. pylori* organism differentially alters the cell cycle compared to non-gastric *E. coli* or *C. jejuni* [17,20,21]. The mechanisms by which either a heterogeneous mixture of bacterial products or the organism itself affects the cell cycle is unknown. In addition, little information exists specifically relating the effects of *H. pylori* LPS to either DNA synthesis or to cell cycle parameters.

We therefore decided to evaluate a toxigenic *H. pylori* LPS (84-183) with known effects on ECL cells on both these parameters in the AGS cell line. In addition, a potential role for CD14, as well as the LPS-activated intracellular pathways were investigated.

11.2 Materials and Methods

11.2.1 Materials

All materials, including the toxigenic *E. coli* LPS (serotype 05:55B) were obtained from Sigma Chemical Co. (Cape Town, South Africa). The well-characterized cytotoxic ($VacA^{+}/CagA^{+}$) *H. pylori* LPS (strain 84-183) were kind gifts from M.J. Blaser and G.I. Perez-Perez (Division of Infectious Diseases, Vanderbilt University, Tennessee, USA). LPS from *C. jejuni* subspecies *jejuni*, biotype 1, serotype 18 (obtained from a 3 month old Cape Town girl with profuse diarrhea) was extracted using a micro-adaptation of the phenol/water extraction procedure of Westphal and Jann [361] as described for small numbers of bacteria [362]. The yield was approximately 1% LPS per wet weight of cells. Protein contamination was less than 1% (ranging from 0.1 – 8 ug/ml) and DNA contamination less than 0.3% (ranging from

0.01 – 2.7 ug/ml). ^{3}H-thymidine was obtained from AEC Amersham (Cape Town, South Africa) (37 MBq; 1mCi/ml). The mouse monoclonal antibody, M-M42 against CD14, was obtained from Novocastra Laboratories Ltd., Newcastle-upon-Tyne, UK. The AGS human gastric epithelial cell line (ATCC CRL 1739) was obtained from ATCC, Rockville, Maryland, USA). RPMI-1640 was obtained from Gibco Brl, Cape Town, South Africa, while a tissue-culture antibiotic solution was obtained from Roche Diagnostics, Johannesburg, South Africa.

11.2.2 Experimental design

Experiments were performed with AGS human gastric epithelial cells (ATCC CRL 1739), and LPS from the *H. pylori* reference strain 84-183 and as negative controls, LPS from *E. coli* and *C. jejuni*. AGS cells were cultured in RPMI-1640 supplemented with 10% FBS and 10ug/ml antibiotic solutions in 180 ml tissue culture flasks at 37 °C in 5% CO_2. Cells were fed fresh medium with serum every 3 days and split when sub-confluent. For all DNA synthesis and flow cytometry experiments, cells were synchronized by serum-deprivation for 24 hr as described [20].

11.2.2.1 To evaluate an effect of LPS on DNA synthesis, serum-deprived AGS cells were incubated with either a maximal concentration of spermine (10^{-5} M) (positive control) or with the LPS (84-183, *E. coli* or *C. jejuni*) preparations (10fg/ml - 0.1mg/ml) for 4 hr. Pre-incubation with polymyxin sulfate (1mg/ml) for 10 min was used to evaluate the specificity of the LPS effect [363]. To examine whether the CD14 receptor modulated LPS, we assessed the effect of pre-incubation for 10 min with the specific monoclonal antibody against CD14, M-M42 (1:100-1:1,000) on *H. pylori* LPS (84-183) (0.1mg/ml) stimulated thymidine uptake. Different cell-permeable inhibitors of intracellular second messenger systems were then used to elucidate the signal transduction pathway mediated by LPS stimulation. All inhibitors were co-incubated with a maximal concentration (0.1mg/ml) of LPS (84-183). Rp-8-Br-cAMP, a cAMP antagonist (reported $K_i \sim 10^{-5}$ M) was used in concentrations of 10^{-6} M to 10^{-4} M with the adenylate cyclase activator forskolin (10^{-5} M) as a positive control. Wortmannin, a microbial metabolite found in a variety of fungal species, inhibits phosphatidylinositol-3-kinase (IP_3 kinase; reported $K_i \sim 3 \times 10^{-9}$ M). It was co-incubated with LPS in concentrations ranging from 10^{-8} to 10^{-5} M.

11.2.2.2 For analysis of cell cycle events, serum-deprived AGS cells were incubated with a maximal concentration of spermine (10^{-5} M) (positive control) or with a maximal

concentration (0.1mg/ml) of the different LPS preparations for 4 hr and analyzed flow cytometrically.

11.2.3 Cell proliferation assay by ^{3}H-thymidine incorporation

Exponentially growing cells were harvested and cultured overnight (for 24 hr) in the absence of serum. Thereafter, serum-starved cells were plated in triplicate at 10^4 cells per well in 96-well flat-bottom microculture plates in 200ul of medium containing serum, 1uCi of ^{3}H-thymidine and LPS/products of interest. Plates were incubated for a further 4 hr, cells harvested, using the Titertek cell harvester and radioactive incorporation measured in a Packard Tri-carb 1500 (Bioteknik, Cape Town, South Africa) liquid scintillation counter.

11.2.4 Flow cytometric analysis

For cell cycle parameter analysis, serum-deprived cells (10^4 cells/experiment) were incubated in 200ul of medium containing serum and LPS (100ug/ml) for 4 hr. Thereafter, cells were incubated with 20 ug/ml propidium iodide, and DNA content was measured using a FACSCaliber (Becton Dickinson, Johannesburg, South Africa) as described [20,364]. Data were plotted using the Cell Quest software (Becton Dickinson); 15,000 events were analyzed for each sample.

11.2.5 Statistical analysis

Results are expressed as mean ± SE. "n" indicates the number of cell studies. Statistical analysis was performed using the two-tailed Student's t-test for paired values as appropriate and p-values < 0.05 were considered significant.

11.3 Results

11.3.1 Effect of LPS on DNA synthesis

The effect of LPS on DNA synthesis in AGS cells was initially measured. Spermine (10^{-5} M) stimulated DNA synthesis as expected (3.13 ± 0.74, $p < 0.01$ vs. control) (Figure 11.1). LPS from 84-183 stimulated DNA synthesis maximally at 1ug/ml (4.43 ± 1.23, $p < 0.05$ vs. control), with an approximate EC_{50} of 20ng/ml. *E. coli* and *C. jejuni* LPS had no significant effect over the concentration range.

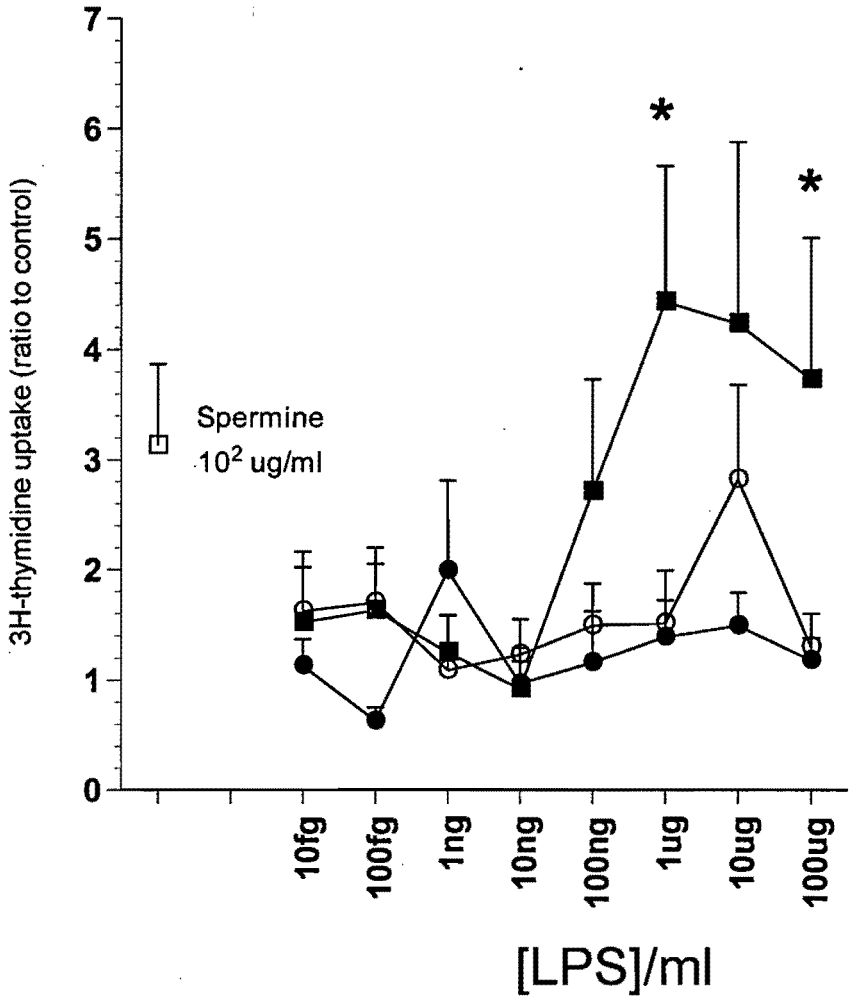


Figure 11.1 Effect of *H. pylori* LPS on DNA synthesis. □ spermine (100ug/ml or 10^{-5} M), O *E. coli* LPS, ● *C. jejuni* LPS, ■ *H. pylori* LPS. All points are expressed as means ± SEM. *p < 0.05 vs. both *E. coli* and *C. jejuni* LPS (n = 6).

11.3.2 Effect of M-M42 on LPS stimulated DNA synthesis

Thereafter, the possibility that CD14 mediated the stimulatory effects of *H. pylori* LPS in the AGS cell was investigated. As in previous experiments, incubation with *H. pylori* LPS (0.1 mg/ml) resulted in a significant stimulation of DNA synthesis (5.08 ± 1.43, $p < 0.01$) (Figure 11.2). Pre-incubation with the monoclonal antibody against CD14, M-M42, for 10 min resulted in a dose dependent reversal of *H. pylori* LPS stimulated DNA synthesis with a maximal effect at a final concentration of 1:100 (1.23 ± 0.16, $p < 0.01$ vs. LPS alone). Pre-incubation of cells with M-M42 alone had no effect on basal DNA synthesis.

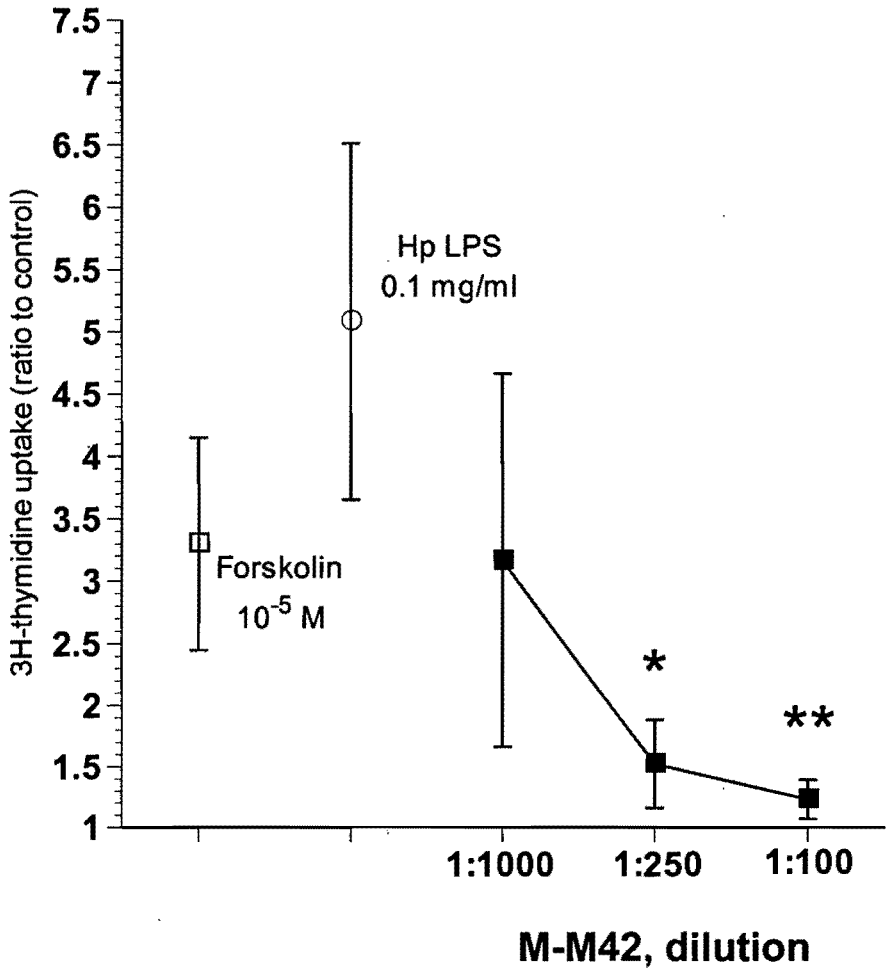


Figure 11.2 Inhibitory effect of M-M42 on *H. pylori* LPS mediated DNA synthesis. □ forskolin 10^{-5} M, O *H. pylori* LPS (0.1 mg/ml), ■ M-M42 + *H. pylori* LPS (0.1 mg/ml). *p = 0.034, **p = 0.008 (n = 6).

11.3.3 Effect of Polymyxin B on LPS stimulated DNA synthesis

The effect of polymyxin B, which specifically binds to lipid A, on LPS mediated DNA synthesis was evaluated. As in previous experiments, incubation with *H. pylori* LPS (0.1 mg/ml) resulted in a significant stimulation of DNA synthesis (5.1 ± 1.4, $p < 0.01$) (Figure 11.3). Pre-incubation with polymyxin B (1 mg/ml) for 10 min resulted in a complete reversal of *H. pylori* LPS stimulated DNA synthesis (0.95 ± 0.2, $p < 0.01$ vs. LPS alone). Pre-incubation of cells with polymyxin alone had no significant effect on basal DNA synthesis.

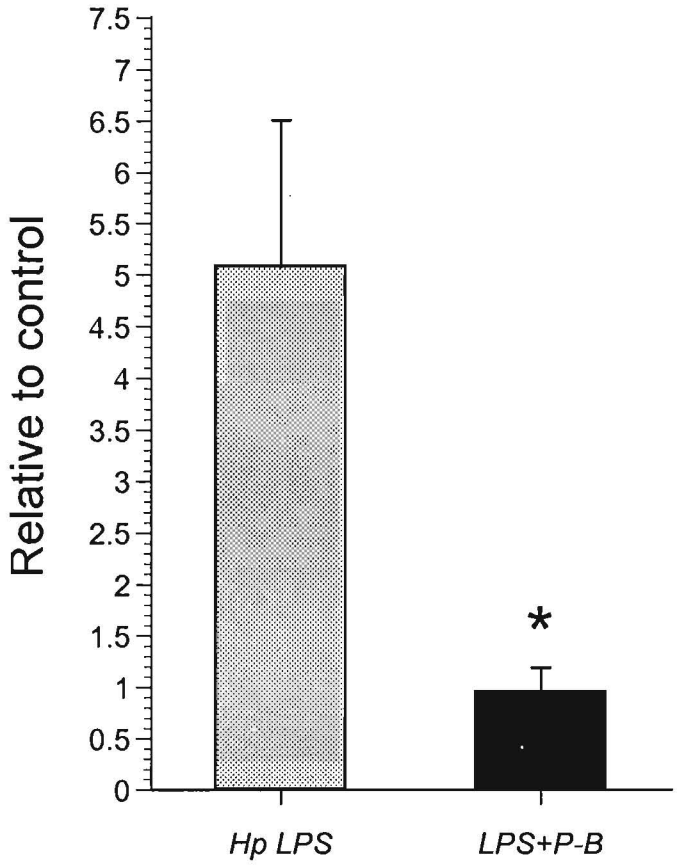


Figure 11.3 Inhibitory effect of polymyxin B on *H. pylori* LPS mediated DNA synthesis. Hp LPS = *H. pylori* LPS (0.1 mg/ml), LPS+P-B = *H. pylori* LPS (0.1 mg/ml) + polymyxin B (1 mg/ml). *$p < 0.01$ ($n = 5$).

11.3.4a Analysis of intracellular pathways mediating LPS effect – IP_3

The effect of the IP_3 kinase inhibitor, Wortmannin, on LPS mediated DNA synthesis was next evaluated. As in previous experiments, incubation with *H. pylori* LPS (0.1 mg/ml) resulted in a significant stimulation of DNA synthesis (5 ± 1.3, $p < 0.01$) (Figure 11.4). Pre-incubation with Wortmannin for 10 min resulted in a reversal of *H. pylori* LPS stimulated DNA synthesis with a maximal effect at 10^{-7} M (2.04 ± 0.6, $p < 0.01$ vs. LPS alone). Pre-incubation of cells with Wortmannin alone had no effect on basal DNA synthesis.

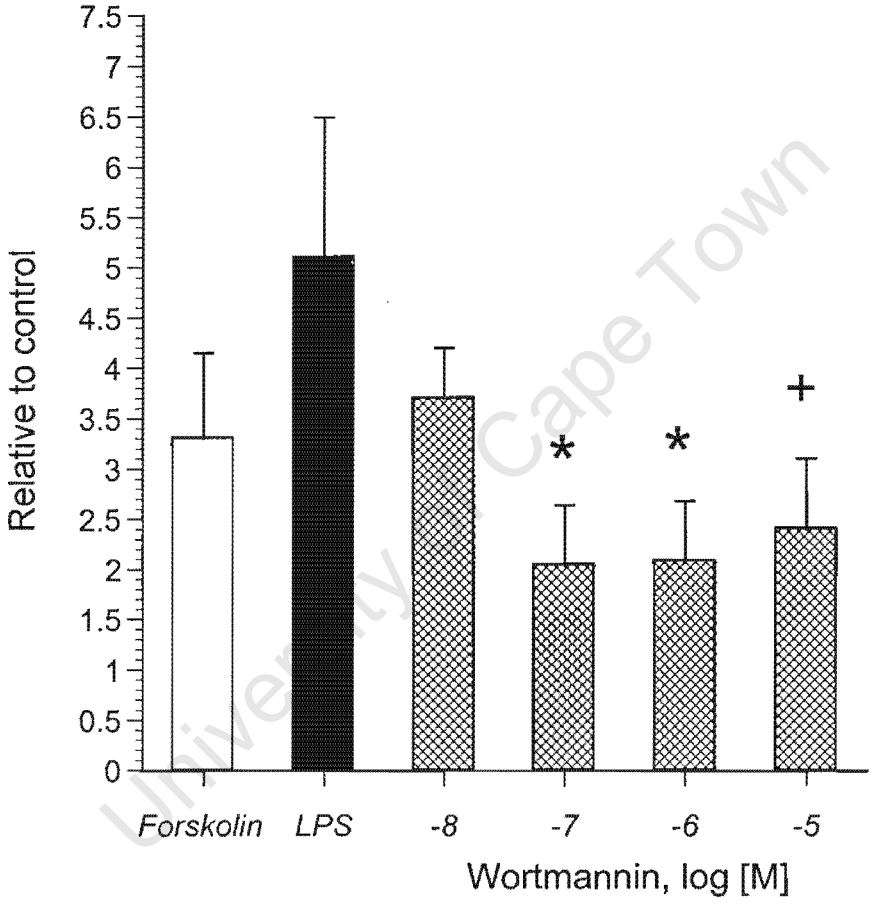


Figure 11.4 Inhibitory effect of Wortmannin on *H. pylori* LPS mediated DNA synthesis. Forskolin = forskolin (10^{-5} M), LPS = *H. pylori* LPS (0.1 mg/ml). Cross-hatched bars = Wortmannin + *H. pylori* LPS (0.1 mg/ml). *$p < 0.03$, +$p = 0.06$, both vs. LPS ($n = 6$).

11.3.4b Analysis of intracellular pathways mediating LPS effect – cAMP

The effect of the cAMP analog, Rp-8-Br-cAMP, on LPS mediated DNA synthesis was thereafter evaluated. As in previous experiments, incubation with *H. pylori* LPS (0.1 mg/ml) resulted in a significant stimulation of DNA synthesis (5.2 ± 1.1, $p < 0.01$) (Figure 11.5). Pre-incubation with Rp-8-Br-cAMP for 10 min resulted in a dose dependent reversal of *H. pylori* LPS stimulated DNA synthesis with a maximal effect at 10^{-4} M (1.31 ± 0.26, $p < 0.01$ vs. LPS alone). Pre-incubation of cells with Rp-8-Br-cAMP alone had no effect on basal DNA synthesis.

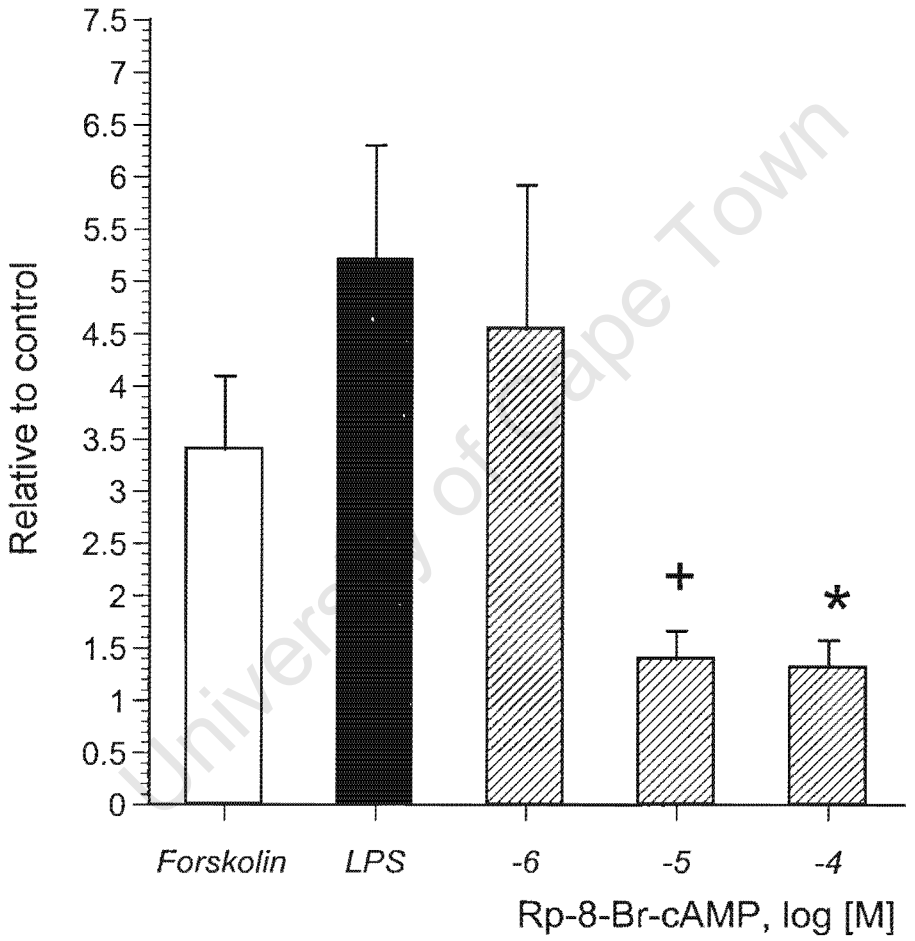


Figure 11.5 Inhibitory effect of Rp-8-Br-cAMP on *H. pylori* LPS mediated DNA synthesis. Forskolin = forskolin (10^{-5} M), LPS = *H. pylori* LPS (0.1 mg/ml), striped bars = Rp-8-Br-cAMP + *H. pylori* LPS (0.1 mg/ml). *$p < 0.01$, +$p < 0.02$, both vs. LPS ($n = 6$).

11.3.5 Effect of LPS on cell cycle parameters

The effect of LPS (0.1 mg/ml) on the AGS cell cycle was next measured. LPS from *C. jejuni* significantly elevated the sub-G_1 fraction (apoptosis) (1.31 ± 0.24, $p < 0.05$ vs. unstimulated) (Figure 11.6). No agent had any significant effect on G_0G_1, but all agents stimulated the $S+G_2M$ fraction ($p < 0.05$ vs. unstimulated). Analysis of the data as a ratio of the growth fraction ($S+G_2M$) to sub-G_1 fraction (Gf/Af) revealed that this ratio was only significantly higher for 84-183 (2.15 ± 0.26, $p < 0.01$ vs. spermine and *C. jejuni*).

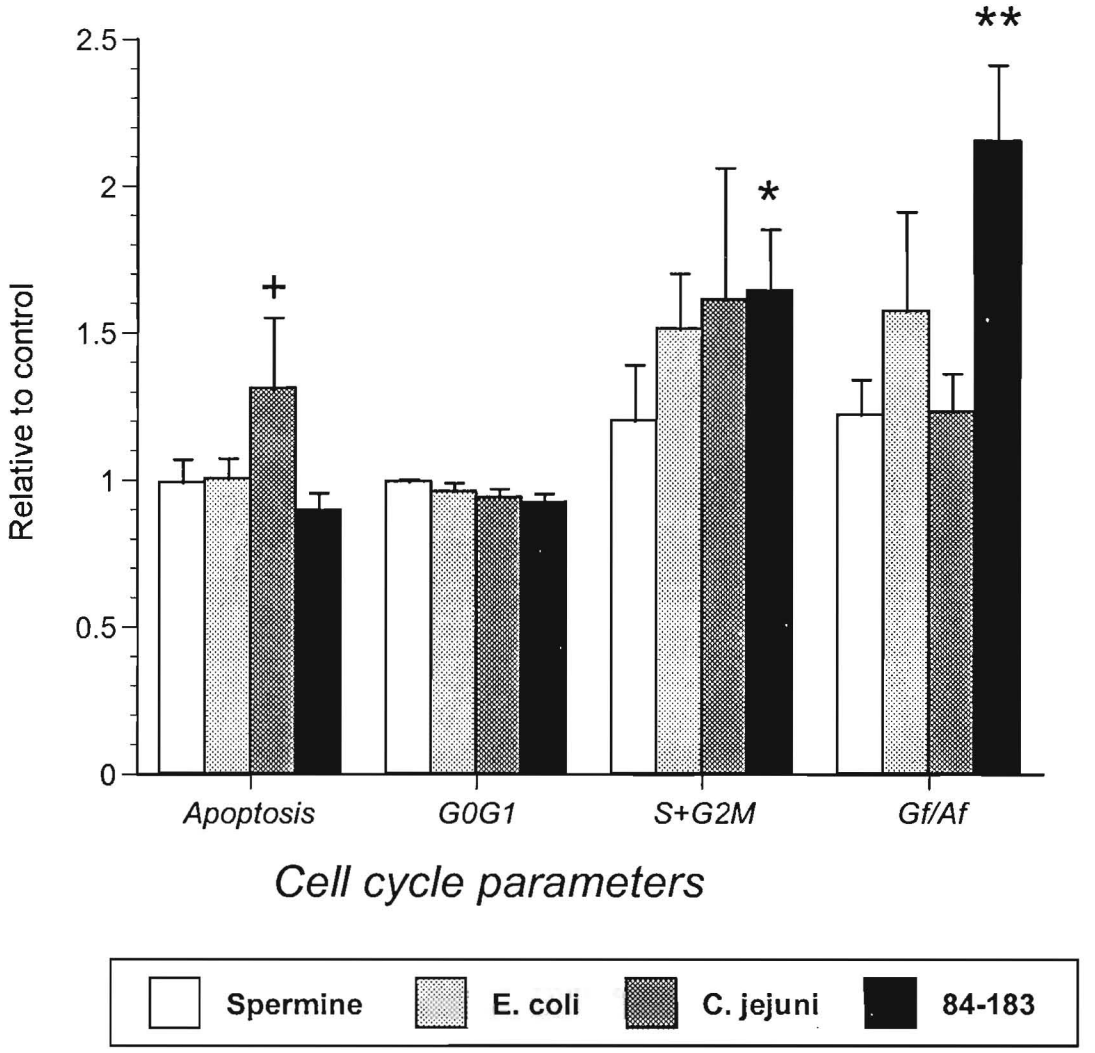


Figure 11.6 Analysis of the effects of LPS on cell cycle parameters. Spermine (10^{-5} M) was utilized as a positive control. All bars are expressed as means ± SEM. $^{+}p = 0.06$ vs. 84-183, $^{*}p = 0.07$ vs. spermine, $^{**}p < 0.01$ vs. spermine and *C. jejuni* (n = 4).

11.3.6 Relationship between DNA synthesis and cell cycle parameters

The relationship between DNA synthesis and both the $S+G_2M$ fraction and the growth fraction ratio was next evaluated (Table 11.1). Linear regression analysis demonstrated significant relationships between DNA synthesis measured by tritiated thymidine uptake and $S+G_2M$ measured flow-cytometrically as well as the calculated Gf/Af ratio. This suggests that measurements of LPS-stimulated DNA synthesis made after 4 hr correlate with alteration in the cell cycle at this time point.

Table 11.1 Relationship between different cell measurements

Parameter	*p-value*	*R*	R^2
DNA synthesis vs. $S+G_2M$	0.0244	0.577	0.333
DNA synthesis vs. Gf/Af	0.0065	0.668	0.446

11.4 Discussion

This data suggest that toxigenic *H. pylori* LPS significantly stimulates both DNA synthesis as well as altering cell cycle parameters in the AGS cell line.

Analysis of DNA synthesis demonstrated that the maximal effective concentration required to stimulate this parameter was 100-fold higher in AGS cells (1 ug/ml) compared to transformed ECL cells (10 ng/ml); a phenomenon which was reflected in the half-maximal concentrations (50 fg/ml vs. 20 ng/ml respectively). Such a difference in effective concentrations suggests that the DNA stimulatory effect in AGS cells may be mediated by a mechanism other than CD14 binding and activation. However, pre-incubation with the specific CD14 antibody, M-M42, reversed the stimulatory effect of *H. pylori* LPS in AGS cells (as it does in ECL cells (cf. Chapter 10)). In addition, pharmacological inhibition of both IP_3 kinase (the Ca^{2+} pathway) as well as the cAMP pathway suggested that LPS mediates G-protein effects in the AGS cell line (CD14 responses are thought to be modulated by either tyrosine kinase activity or redistribution of intracellular G-proteins [269]). The combination of the antibody inhibitor and the intracellular pathway studies, however, suggest strongly that

the LPS effect on the AGS cell is most likely mediated via CD14. It is possible that the differences between this cell line and the ECL cell may reflect differences in receptor number and/or specificity.

The intracellular mechanism by which LPS alters the cell cycle is unknown. CD14 has been demonstrated to co-precipitate with tyrosine kinase activity [365], and activation of the receptor results in rapid phosphorylation of p38 [366]. It is possible that activation of a tyrosine kinase pathway, as noted for TGFα in ECL cells [345], may play a role in proliferation, but the inhibitory effects of Rp-8-Br-cAMP and Wortmannin suggest a different pathway. CD14 may also be coupled to G-proteins [367] and it would appear likely that activation of stimulatory G-subunits modulates the proliferative response in AGS cells. This would not be dissimilar to the effects of gastrin, which is known to affect ECL cell proliferation via IP_3 and ras/MAPK/c-*fos* and c-*jun* activation [368]. In AGS cells, use of the cAMP analog appeared to be more inhibitory than IP_3 and, while suggesting this may be the dominant pathway driving DNA synthesis (almost complete inhibition [95%] versus ~80% with Wortmannin), this could simply reflect the different uptake of these agents in this cell system. It is known for example that the latter agent (IP_3 inhibitor/Ca^{2+} pathway) possesses a low potency in intact cell systems [369]. Interestingly, differentiation rather than proliferation is associated with the protein kinase C (PKC) pathway in the Mono Mac 6 cell line [356].

Polymyxin B, by binding to lipid A, is a well known inhibitor of the activation properties of *Enterobacteriaceae* LPS [363]. Preincubation with this agent markedly inhibited the ability of *H. pylori* LPS to induce DNA synthesis. This suggests that the basis for activation of the AGS cell line by *H. pylori* LPS may be lipid A mediated, in spite of the marked differences in lipid A structure compared with the *Enterobacteriaceae* [258]. The ability to activate DNA synthesis may therefore be a function of conserved core structures. The differences in DNA synthesis noted for the different bacterial LPS species studied suggests, however, that *H. pylori* LPS may be unique in its ability to promote this parameter.

Analysis of cell cycle events at 4 hr with a maximal concentration of LPS (0.1 mg/ml) demonstrated no statistically significant effect on the sub-G_1 (apoptotic) fraction, except for LPS from the toxigenic *C. jejuni* isolate. Previous studies have demonstrated that *C. jejuni* does not alter gastric epithelial cell viability or apoptosis *in vitro* [17,370]. These variances in results may reflect differences in both the study designs and methodologies used. Other studies, with co-cultures of *H. pylori* and AGS cells could demonstrate no significant alterations in apoptosis after 6 hr [20]. A second study demonstrated that the most marked

alterations in cell viability and apoptosis were noted only after 72 hr [21]. Injection, *in vivo*, of 0.5-2 mg/ml LPS from the pathogenic *H. pylori* isolate (ATCC 11637) in Sprague-Dawley rats resulted in gastric apoptosis [271]. This effect, seen after 48 hr, must have been non-specific since LPS from ATCC 11637 has previously been demonstrated to have a mitogenic effect in 18 hr mouse spleen cultures [371]. It would appear that the time frame of the present study (4 hr incubation with LPS) may be too short to evaluate any significant apoptotic event, although this appears unlikely given the apoptotic observations in a co-culture study [20].

All LPS preparations in this study had stimulatory effects on the $S+G_2M$ fraction, but not the G_0G_1 phase. An effect of *H. pylori* inducing a block at the G_1 phase has been mooted [20]. This, along with a decrease in the S-phase, was only seen after 48 hr of bacterial co-incubation. The present study suggests that LPS may have a direct effect on DNA synthesis rather than resulting in a G_1 block. Alterations in the balance of proliferation to apoptosis (proliferation > 1.0, apoptosis < 1.0) have been demonstrated to occur during gastrin mediated gastric mucosal alterations *in vivo* [372]. Analysis of this ratio in the present study demonstrated that this was most significant for toxigenic *H. pylori* LPS and after 4 hr these effects on AGS cells were most likely to be proliferative. Such an observation was supported by the strong relationship between this ratio and DNA synthesis ($r = 0.67$, $p = 0.008$). It is possible that DNA synthesis (measured by thymidine uptake) may be a surrogate marker for cell cycle progression (proliferation).

11.5 Conclusion

A model system examining the effects of LPS on a biologically relevant gastric adenocarcinoma cell has been demonstrated. This study has shown that toxigenic *H. pylori* LPS significantly and positively alters both DNA synthesis and cell cycle parameters in the gastric epithelial AGS cell *in vitro*. These effects, as in the ECL cell, appear to be mediated by CD14 with the respective intracellular alterations.

Chapter 12

Analysis of the effects of LPS from clinical isolates on transformed gastrointestinal cells

12.1 Introduction

Toxigenic *H. pylori* LPS positively alters DNA synthesis and the cell cycle in the gastric AGS cell line *in vitro* (cf. Chapter 11). Previous studies have shown that *cagA*$^+$ strains selectively enhance proliferation and attenuate apoptosis compared with *cagA*$^-$ strains [373]. A second study from the same group demonstrated that a diminished AGS cell viability, progression to the G_2M phase and apoptosis, associated with co-incubation with particular *cag*$^+$ strains, were mediated in part by expression of *vacA* and genes in the *cag*PAI (particularly *cagE*) [21]. Three other studies have produced differing views. The ability of *H. pylori* to alter apoptosis [17,20,374] or proliferation [17,20] did not appear to be related to strain variation when analyzed by *cagA* status and toxin production. Nevertheless, *H. pylori* colonization increases gastric mucosal apoptosis [105,106,375] but epithelial cell proliferation rates in gastric tissue from people harboring the organism have been either reduced or enhanced in various studies [373,376,377].

The role of specific bacterial characteristics to explain this variation in induction of cell growth or death remains a valid and attractive hypothesis. It was therefore decided to examine the effects of LPS from South African clinical isolates with defined virulence profiles on both DNA synthesis and the cell cycle in the established AGS cell system.

12.2 Materials and Methods

12.2.1 Materials

All materials, including *E. coli* LPS (serotype 05:55B) we obtained from Sigma Chemical Co. (Cape Town, South Africa). The well-characterized cytotoxic (VacA$^+$/CagA$^+$) *H. pylori* LPS (strain 84-183) were kind gifts from M.J. Blaser and G.I. Perez-Perez (Division of Infectious Diseases, Vanderbilt University, Tennessee, USA). LPS from *C. jejuni* (cf. Chapter 11) was used as a non-gastric control. ^{3}H-thymidine was obtained from AEC Amersham (Cape Town, South Africa) (37 MBq; 1mCi/ml). The mouse monoclonal antibody, M-M42 against CD14, was obtained from Novocastra Laboratories Ltd., Newcastle-upon-Tyne, UK. The AGS human gastric epithelial cell line (ATCC CRL 1739)

was obtained from ATCC, Rockville, Maryland, USA). RPMI-1640 was obtained from Gibco Brl, Cape Town, South Africa, while a tissue-culture antibiotic solution was obtained from Roche Diagnostics, Johannesburg, South Africa.

12.2.2 H. pylori strains

Twelve *H. pylori* strains were selected from a larger population of isolates that had been previously described as part of an ongoing study designed to evaluate the relationship between virulence factors and clinically significant disease in populations from South Africa (c.f. Chapters 5,6,7,8). Because we sought to evaluate the potential importance of virulence-associated genes (i.e. *cagA*, 3'-fragment length of *cagA*, 5'-region of *cag*PAI, *vacA*, *iceA*) in the modulation of cell cycle events, we selected strains varying in these parameters (Table 12.1).

Table 12.1 Characteristics of South African clinical *H. pylori* strains

Strain	***vacA***	***cagA-3'***	***cag*PAI**	***iceA***	**Pathology**
450	s2m2	< 600	*cagIIp*	0/2	gastritis
501	s1bm2	< 600	*cagIIp*	1/2	gastritis
508	s1bm1	600-700	*cagIIp*	0/0	gastritis
548	s1bm1	600-700	*cagIIp*	1/2	gastritis
218	s1bm2	< 600	complete	1/2	PUD
234	s1bm1	< 600	complete	1/0	PUD
240	s1bm1	> 700	*cagIIp*	1/0	PUD
*442	s1bm1	< 600	complete	1/0	PUD
Ca42	s1bm2	> 700	complete	1/2	GCa
Ca45	s1bm1	600-700	complete	1/0	GCa
Ca84	s1bm1	600-700	complete	0/2	GCa
Ca90	s1bm2	600-700	*cagIIp*	0/2	GCa

PUD = peptic ulcer disease, GCa = gastric adenocarcinoma, *cagIIp* = partial *cag*PAI (missing the 5'-region of *cagII*)

*442 = the structure of the lipopolysaccharide side chains are known [378]

12.2.3 Preparation of LPS

H. pylori which conformed to standard phenotypic and biochemical criteria were harvested, washed once with normal saline, centrifuged at 10,000g for 10 min and stored at –20 °C. After thawing, the LPS was extracted using a microadaptation of the phenol/water extraction procedure of Westphal and Jann [361] as described for small numbers of bacteria [362]. The yield was approximately 1% LPS per wet weight of cells. In total, protein contamination was less than 1.25% (ranging from 0.1 – 12.5 ug/ml) and DNA contamination less than 0.25% (ranging from 0.01 – 2.3 ug/ml).

12.2.4 Experimental design

Experiments were performed with AGS human gastric epithelial cells (ATCC CRL 1739), and LPS from the South African *H. pylori* strains, with 84-183 as a positive control and as non-gastric controls, LPS from *E. coli* and *C. jejuni* (cf. Chapter 11). AGS cells were cultured in RPMI-1640 supplemented with 10% FBS and 10ug/ml antibiotic solution in 180 ml tissue culture flasks at 37°C in 5% CO_2. Cells were fed fresh medium with serum every 3 days and split when sub-confluent. For all DNA synthesis and flow cytometry experiments, cells were synchronized by serum-deprivation for 24 hr.

12.2.4.1 Exponentially growing cells were harvested and cultured overnight (for 24 hr) in the absence of serum. Thereafter, serum-starved cells were plated in triplicate at 10^4 cells per well in 96-well flat-bottom microculture plates in 200ul of medium containing serum, 1uCi of ^{3}H-thymidine and LPS (0.1 mg/ml). Plates were incubated for a further 4 hr, cells harvested, using the Titertek cell harvester and radioactive incorporation measured in a Packard Tri-carb 1500 (Bioteknik, Cape Town, South Africa) liquid scintillation counter.

12.2.4.2 For cell cycle parameter analysis, serum-deprived cells (10^4 cells/experiment) were incubated in 200ul of medium containing serum and LPS (0.1mg/ml) for 4 hr. Thereafter, cells were incubated with 20 ug/ml propidium iodide, and DNA content was measured using a FACSCaliber (Becton Dickinson, Johannesburg, South Africa) as described [20,364]. Data were plotted using the Cell Quest software (Becton Dickinson); 15,000 events were analyzed for each sample.

12.2.5 Statistical analysis

Results are expressed as mean ± SE. "n" indicates the number of cell studies. Statistical analysis was performed using the two-tailed Student's t-test for paired values as appropriate and p-values < 0.05 were considered significant.

12.3 Results

12.3.1 DNA synthesis, cell cycle parameters and the cagPAI

The effect of LPS (0.1mg/ml) from twelve different *H. pylori* isolates on DNA synthesis and cell cycle parameters in AGS cells was measured. LPS from isolates with an intact *cag*PAI stimulated DNA synthesis (3.7 ± 0.87) more than from isolates with a partial *cag*PAI (1.18 ± 0.2) ($p < 0.015$). This was also significantly higher than from *E. coli* ($p = 0.04$) and *C. jejuni* ($p < 0.02$) (Figure 12.1). Deletions in the *cag*PAI were not associated with any alterations in the sub-G_1 fraction and the G_0/G_1 fraction but LPS from isolates with an intact *cag*PAI increased ($p = 0.036$) the S+G_2M fraction (1.42 ± 0.08) compared to a partial *cag*PAI (1.22 ± 0.05). The growth fraction tended to be higher in the intact PAI group ($p = 0.07$).

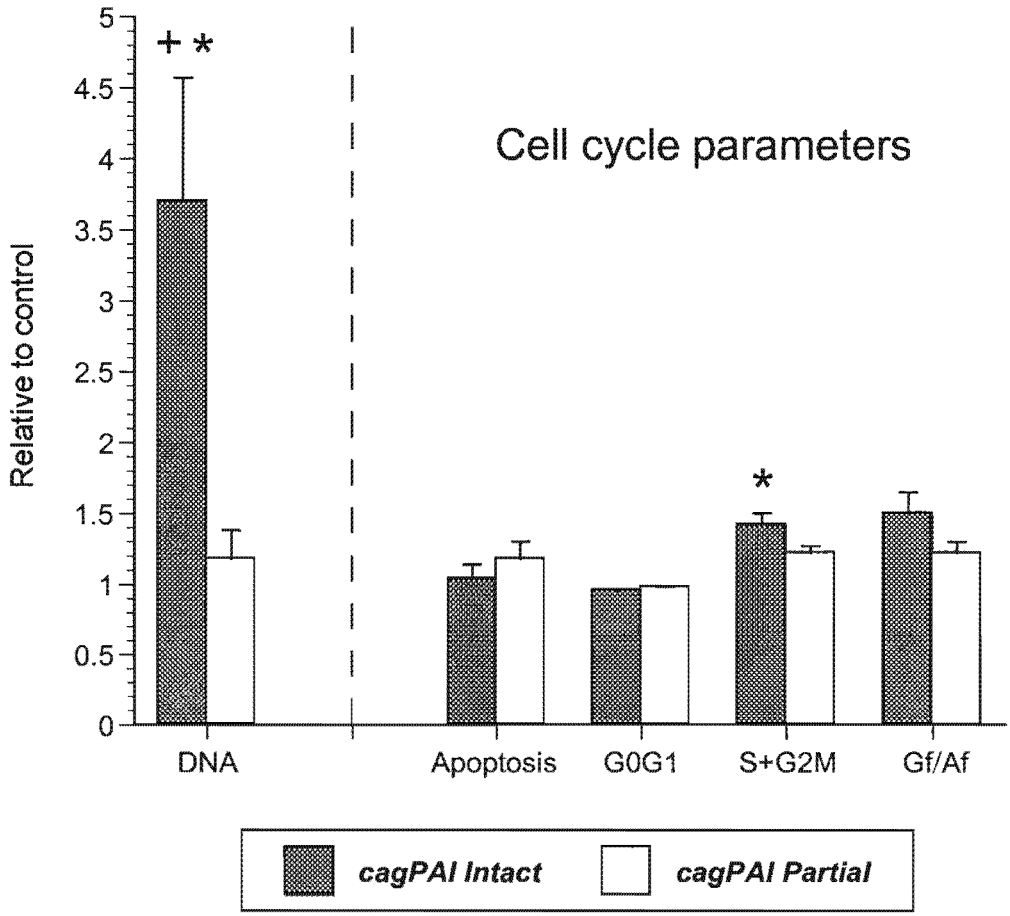


Figure 12.1 Effect of LPS on DNA synthesis and cell cycle parameters. LPS from *E. coli* and *C. jejuni* were used as non-gastric controls, while 84-183 was a gastric control. Intact = complete *cag*PAI, partial = *cag*PAI with deletions in the 5'-region (*cagII*). Gf/Af = the ratio of the growth fraction to the apoptotic fraction. $^{*}p < 0.03$ vs. partial, $^{+}p < 0.05$ vs. *E. coli* (1.3 ± 0.3), and *C. jejuni* (1.18 ± 0.2) (n = 4).

12.3.2 DNA synthesis, cell cycle parameters and cagA-3' length

LPS from isolates with the largest *cagA3'*-fragment length (> 700 bp) significantly stimulated DNA synthesis (6.15 ± 2.3, p = 0.0005) compared to LPS from isolates with a small (< 600 bp) *cagA3'*-fragment length (1.26 ±0.16) (Figure 12.2). LPS from isolates with an intermediate fragment length (600-700 bp) had a similar effect (3.6 ± 0.9, p < 0.003 vs. < 600 bp). *cagA3'*-fragment length had no statistically significant effect on cell cycle parameters.

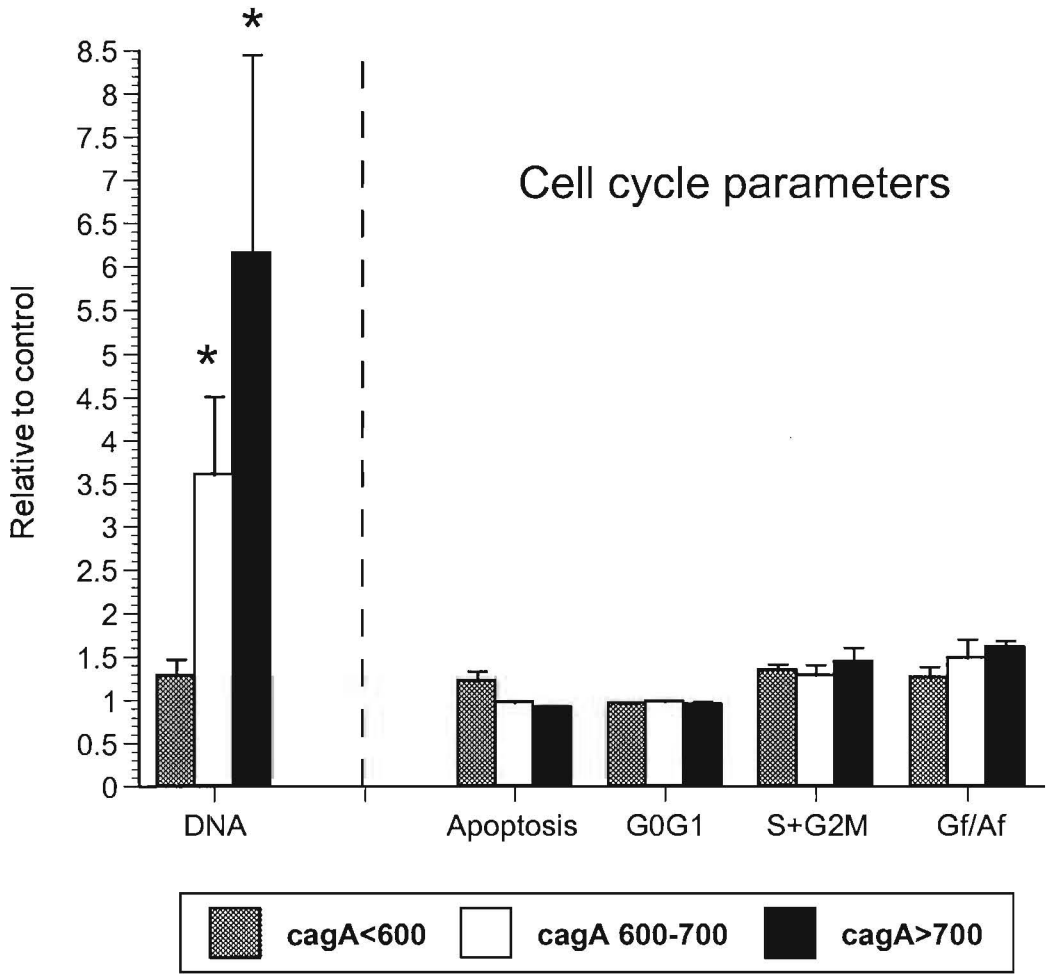


Figure 12.2 Effect of LPS on DNA synthesis and cell cycle parameters. LPS from *E. coli* and *C. jejuni* were used as non-gastric controls, while 84-183 was a gastric control. cagA<600 = isolates with shortest fragment length, 600-700 = isolates with an intermediate fragment length, cagA>700 = isolates with the longest fragment length. $^{*}p < 0.003$ vs. cagA<600 and < 0.03 vs. *E. coli* (1.3 ± 0.3), and *C. jejuni* (1.18 ± 0.2) (n = 4).

12.3.3 vacA alleles, DNA synthesis and cell cycle parameters

LPS from isolates with the *vacA* s1b/m1 type stimulated DNA synthesis (2.56 ± 0.6) significantly more than from *C. jejuni* (1.18 ± 0.2, p = 0.037) (Figure 12.3). The *vacA* s1bm2 tended to be higher than *C. jejuni* (p = 0.08) while s2m2 the smallest effect. Because the latter was only one isolate it was excluded from formal statistical analysis. Analysis of cell cycle parameters demonstrated that LPS from isolates with *vacA* s1bm1 had a significantly lower effect on the sub-G_1 fraction (0.99 ± 0.03) than LPS from *C. jejuni* (1.31 ± 0.24, p = 0.04) and isolates with *vacA* s1/bm2 (1.27 ± 0.19, p = 0.048). No other alterations in the cell cycle were noted.

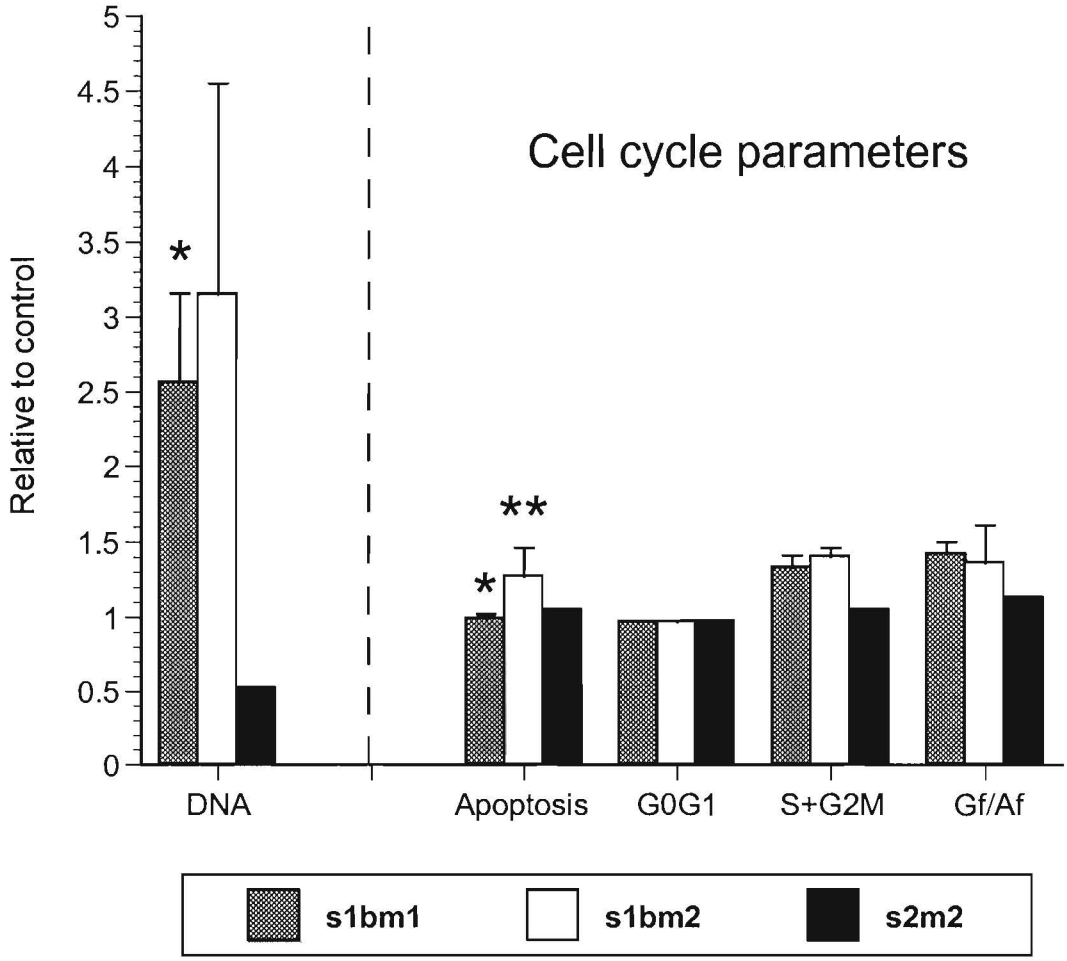


Figure 12.3 Effect of LPS on DNA synthesis and cell cycle parameters. LPS from *E. coli* and *C. jejuni* were used as non-gastric controls, while 84-183 was a gastric control. $^{*}p < 0.05$ vs. *C. jejuni*, $^{**}p < 0.05$ vs. *vacA* s1b/m1, (n = 4).

12.3.4 iceA alleles, DNA synthesis and cell cycle parameters

LPS from isolates with *iceA1*, or *iceA1/2* tended to increase DNA synthesis compared to *C. jejuni* (1.18 ± 0.2, both $p < 0.06$) but not against *E. coli* (1.3 ± 0.3) (Figure 12.4). No differences in stimulation of DNA synthesis were, however, noted within the *iceA* groups. None of the *iceA* genotypes had any effect on the sub-G_1, G_0G_1-fraction or S+G_2M fractions. No differences in the growth fraction were noted. The null *iceA* genotype was only present in one isolate was excluded from formal statistical analysis

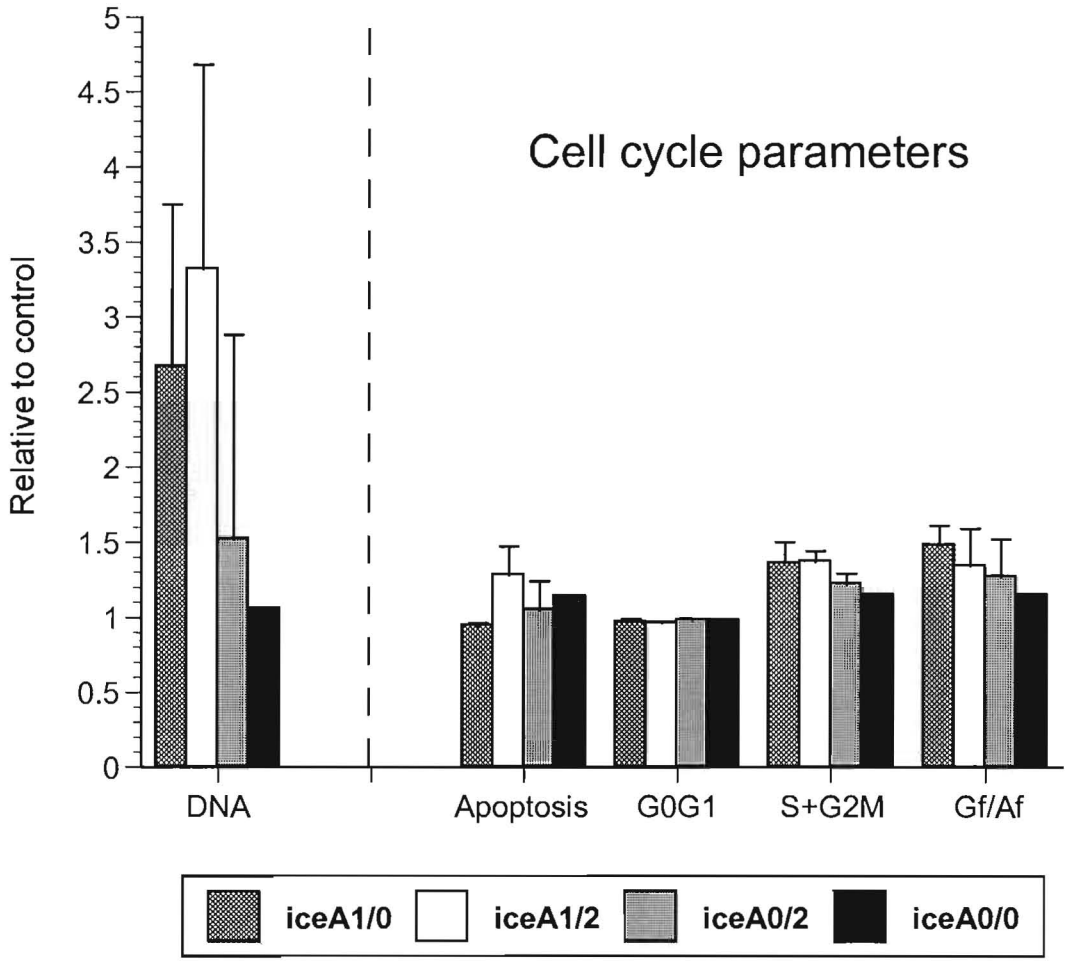


Figure 12.4 Effect of LPS on DNA synthesis and cell cycle parameters. LPS from *E. coli* and *C. jejuni* were used as non-gastric controls, while 84-183 was a gastric control (n = 4).

12.3.5 Clinical presentation, DNA synthesis and cell cycle parameters

LPS from patients with gastric adenocarcinoma (4.61 ± 1.6) significantly stimulated DNA synthesis more than from patients with gastritis alone (1.21 ± 0.27, $p < 0.04$), peptic ulcer disease (1.49 ± 0.13, $p = 0.05$), *E. coli* (1.3 ± 0.3, $p = 0.04$) and *C. jejuni* (1.18 ± 0.2, $p < 0.02$) (Figure 12.5). Analysis of cell cycle parameters demonstrated that LPS from patients with gastric adenocarcinoma had a tendency to a lower apoptotic fraction (0.99 ± 0.04, $p = 0.07$ vs. gastritis) and significantly higher growth ratio (1.36 ± 0.08) compared to gastritis (1.13 ±0.09, $p < 0.05$). In addition, adenocarcinoma isolates had a higher G_0G_1 fraction (1.0 ± 0.01, $p = 0.04$ vs. *E. coli* 0.94 ± 0.03). Gastritis isolates also tended to have a lower $S+G_2M$ fraction (1.19 ± 0.07) compared to PUD isolates (1.43 ± 0.11, $p < 0.06$) but this was not formally significant.

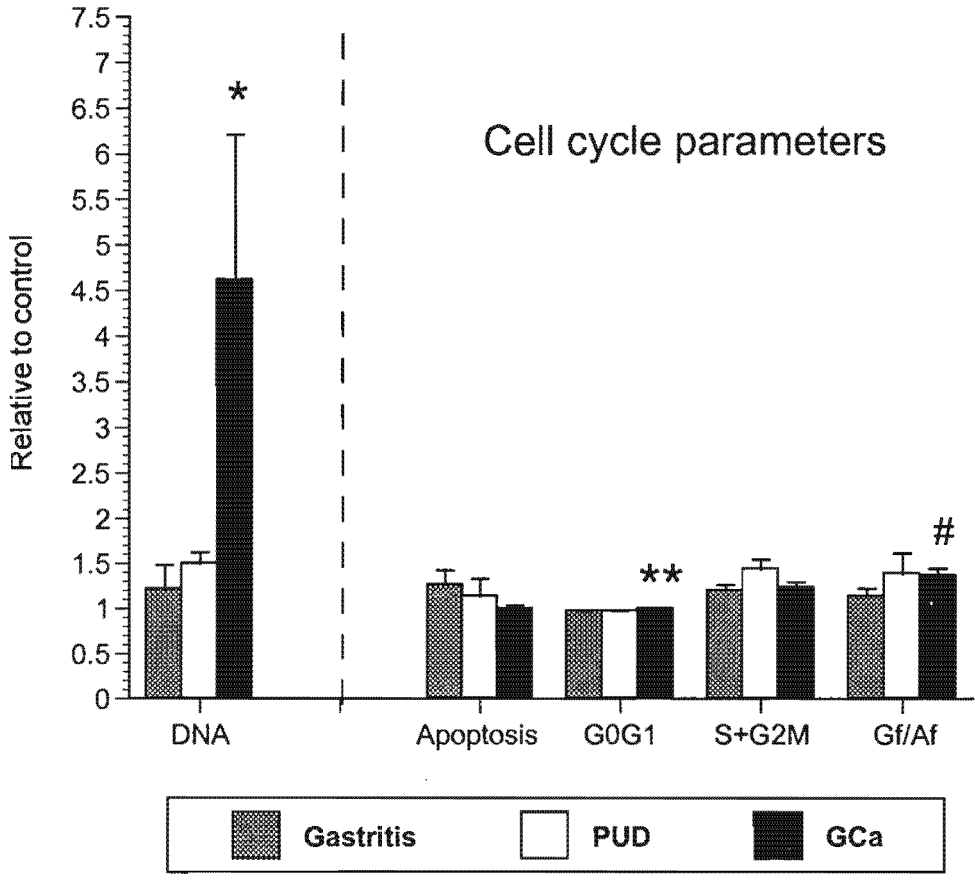


Figure 12.5 Effect of LPS on DNA synthesis and cell cycle parameters. LPS from *E. coli* and *C. jejuni* were used as non-gastric controls, while 84-183 was a gastric control. PUD = peptic ulcer disease, GCa = gastric adenocarcinoma. $^{*}p < 0.05$ vs. *E. coli*, *C. jejuni*, Gastritis, PUD, $^{**}p < 0.05$ vs. *E. coli*, $^{\#}p < 0.05$ vs. gastritis (n = 4).

12.4 Discussion

The present study analyzes the effects of LPS from twelve clinically defined South African *H. pylori* isolates in an established and validated system. The data suggest strongly that LPS from isolates with specific virulence fingerprints are associated with alterations in both DNA synthesis as well as the cell cycle. The alternative explanation that the cell cycle effects may be due to contamination of LPS with virulence proteins requires some attention, although this appears unlikely given the stringent methodology to isolate LPS (phenol extraction and boiling) which would denature proteins, as well as the estimated nanomolar concentrations of any contaminant. The cell cycle effects noted are therefore most likely due to the LPS molecule itself.

LPS derived from strains with an intact PAI, compared to a PAI with gene losses in the 5'-region of the island, was significantly associated with increases in DNA synthesis, the $S+G_2M$ fraction and the calculated growth fraction. These results are similar to one other study which examined, by flow cytometry, the relationship between genes in the *cag*PAI and the cell cycle in the AGS cell [21]. This study demonstrated that strains with a *cagE* mutation (in *cagI*) significantly attenuated cell cycle progression at 6 hr and decreased apoptosis with enhanced cell viability at 24 hr. It is possible that genes within the *cag*PAI are associated with the function of the bacterial LPS, either in terms of the construction of LPS or export of this cell wall constituent [214]. All isolates were *cagA*$^+$ in the present study, so it was not possible to analyze a relationship between the presence or absence of the gene and the effects of LPS. It was, however, possible to investigate the relationship between differences in the length of *cagA* and LPS. An increase in the size (> 600 bp) of the *cagA*3'-gene, which has been previously associated with gastric adenocarcinoma in Japanese isolates [218] and in South African isolates (cf. Chapter 6), was linked with an increase in DNA synthesis in AGS cells but not to any significant alterations in the cell cycle. While three previous studies have shown no relationship between the gene *cagA* and alterations in the AGS cell [17,20,374], the effect of variability in the 3'-region of this gene was not reported. Interestingly, there appears to be a linkage between Lewis expression (particularly Le^y) and the *cagA* gene [10]. The authors suggest that the *cagA* gene product may alter either Le^y expression or localization. It is possible that an extended *cagA*3'-fragment length (which alters the hydrophilicity and immunogenicity of CagA) may result in LPS with differing Lewis determinants.

In this study, LPS from isolates with the *vacA* s1b subtype was associated with an increase in DNA synthesis. This is consistent with the 6 hr results from Peek et al [21], which

demonstrated that *vacA* s1b was more strongly associated with an increase in G_2M than either subtype s1a or s2. Analysis of alterations in apoptosis indicate that the *vacA* s1b/m2 subtype appeared to be more apoptotic than s1b/m1 subtype in the present study. This suggests that differences in the mid-region may be correlated with LPS-mediated apoptosis. This is not, however, confirmed by the co-incubation studies, which demonstrated a stronger relationship with the *vacA* signal sequence subtype rather than mid-region [21]. Interestingly, the effects of the *iceA* alleles (the *iceA1* allele is associated with peptic ulcer disease [185,223] and gastric adenocarcinoma (cf. Chapter 7) while *iceA2* is associated with gastritis alone [185]) on LPS demonstrated that there was no association with any statistically significant alterations in either DNA synthesis or cell cycle parameters in the present study.

The chemical structure of one of the LPS (strain 442) is known [378]. The O-side chain of LPS in this strain is terminated with Le^x moieties, and it appears similar to P466, whose LPS carries the cancer-associated type 2 sialyl Le^x blood group antigen [379]. Strain 442 is isolated from a patient with peptic ulcer disease and is characterized by the *vacA* s1bm1 allele with an intact *cag*PAI (*cagA* 3' < 600 bp) as well as being *iceA1*$^+$. LPS from this isolate is also associated with increased pepsinogen secretion [260,380]. In this study, the stimulation of DNA synthesis and cell cycle effects of LPS from strain 442 are similar to those of the other LPS isolated from patients with PUD. This suggests that the marked stimulation of DNA synthesis in the LPS isolated from patients with gastric cancer (cf. Figure 12.5) may not be due to the presence of Le^x structures. It is possible that either the O-side chain of the latter isolates may terminate in other Lewis moieties or else that there may be differences in the lipid A structure which results in these effects. The finding that polymyxin B reverses the DNA stimulatory effect of LPS from *H. pylori* 84-183 (cf. Figure 11.3) suggests that lipid A, rather than the O-side chain of LPS, may be associated with biological activity. This is supported by results from a separate study that demonstrated that different biological activities appeared to directly correlate with the molecular shape of lipid A from Gram-negative bacteria [381].

12. 5 Conclusion

In summation, the data suggest that specific virulence fingerprints are associated with alterations in the cell cycle and that LPS may be a surrogate marker for the effects of these fingerprints (Figure 12.6). In addition, this association would also appear to be clinically relevant. Gastric adenocarcinoma isolates have an increased DNA synthesis, lower apoptosis,

higher S+G_2M and an increased growth fraction. PUD isolates did not have an increased DNA synthesis but certainly an increased S+G_2M, which was not consistently reflected by the calculation of the growth fraction. In contrast, gastritis isolates have lower DNA synthesis, increased apoptosis, and decreases in S+G_2M as well as in the growth fraction. These different effects of LPS may be potentially related to differences in lipid A structures.

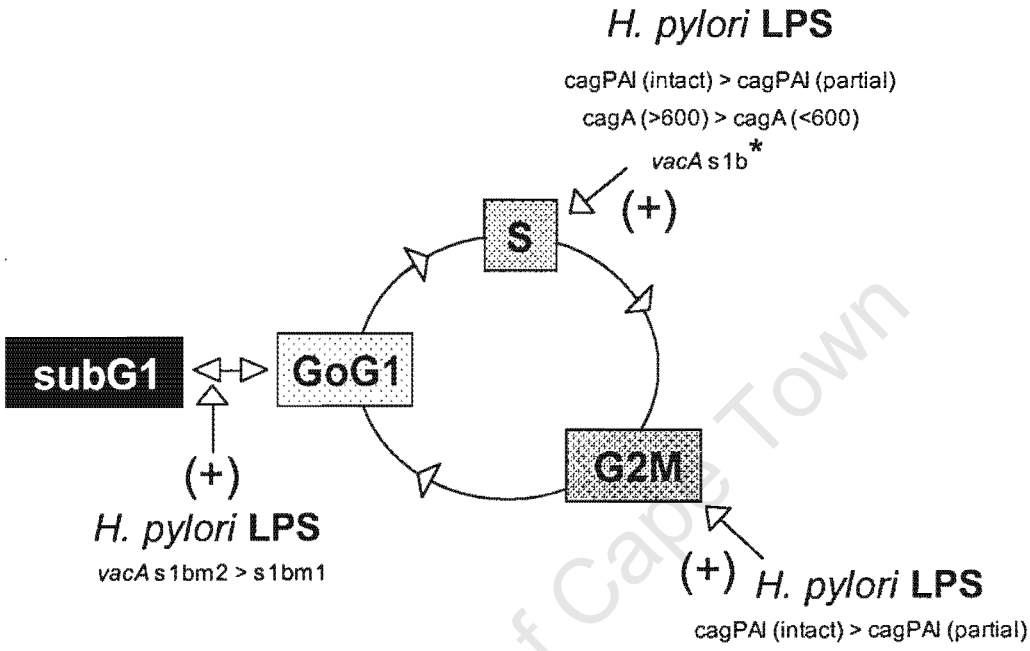


Figure 12.6 Summation of the effects of LPS from South African *H. pylori* isolates with different virulence fingerprints. LPS from isolates with the *vacA* s1bm2 allele stimulated entry into the apoptotic (sub-G_1) fraction more than the *vacA* s1bm1 allele. LPS from isolates with an intact *cag*PAI and a *cagA* 3'-fragment length > 600 bp stimulated DNA synthesis more than isolates with either a partial *cag*PAI or *cagA* < 600 bp. **vacA* s1b was also associated with an increase DNA synthesis. Entry into the G2M phase was increased by LPS from isolates with an intact *cag*PAI compared to a partial *cag*PAI.

Chapter 13.

Concluding summary & directions for future research

Investigations and analysis of *Helicobacter pylori* virulence and its relationship to pathobiology have been conducted primarily in the developed world. South Africa has a high level of *H. pylori* infection, but little is known about the prevalence of either clinically significant disease or the virulence of the organism. This study, using isolates derived mainly from patients in the Western Cape, set out to investigate the virulence profile of these clinical isolates and to measure their virulence potential *in vitro*.

A virulence profile provides a set of markers (genes or gene products) which denote virulence if they segregate with disease. Analysis of a number of candidate virulence genes in South African *H. pylori* isolates was undertaken in order to determine whether a "virulent fingerprint" existed in these organisms.

The low cytotoxic *vacA* s1b allele was widespread (as in Portugal and Brazil), and the more cytotoxic *vacA* s1a almost completely absent. The non-cytotoxic *vacA* s2 allele was exclusively found in patients with gastritis alone. It is possible that the high prevalence of the *vacA* s1b low-cytotoxic allele may modulate disease expression in the Western Cape. Both peptic ulcer disease and gastric cancer isolates had the same *vacA* s1b fingerprint as well as a similarly high distribution of the *vacA* m1 allele. It is apparent therefore that *vacA* alleles do not segregate between these two diseases which have very different etiologies.

The gene, *cagA*, was identified in 100% of isolates (as in Asian countries) but differences in its genetic product (an increase in the length of the 3' region) identified South African strains associated with gastric cancer. Interestingly, these differences expressed themselves both in alterations in protein secondary structure (an increased number of surface exposed regions) as well as in the potential number of tyrosine phosphorylation sites. This suggests that differences in protein structure may be important in determining antigenicity, and it is possible that CagA from South African gastric cancer patients is potentially more antigenic. Transduction of the signaling effects of CagA is mediated only via tyrosine phosphorylation. It is possible that CagA from cancer strains may preferentially affect pro-proliferative signaling pathways in epithelial gastric cells because of the increased number of potential tyrosine sites. CagA from peptic ulcer disease isolates tended mostly to be of the

shorter variant which is differently phosphorylated. Analysis of specific genetic markers of the *cag*PAI suggested that the functionally significant *cagE* product, which is important for the translocation and phosphorylation of CagA, was absent in a number of isolates from patients with gastritis alone. Genes in the 5'-region of the island also tended to be "lost" or deleted in a number of strains from patients without clinically significant disease. This work suggests a supportive role for these gene products in pathogenesis. Indeed, an intact *cag*PAI is a prerequisite for CagA translocation and phosphorylation. It is possible that the alterations in the *cag*PAI noted in patients with gastritis alone may make the organisms "non-virulent." Interestingly, strains from patients with both peptic ulcer disease and gastric adenocarcinoma had intact islands, yet the diseases have different etiologies. The major difference between these two groups was in the length of CagA. It is attractive to hypothesize that while CagA may be transported into epithelial cells in all cases, its coupling with secondary messenger systems may differ depending on its size. This may potentially contribute to the different spectrum of diseases noted.

Alleles and structural variants of the gene, *iceA*, tended to differ between disease groups. Specifically *iceA1* appeared to be a marker for gastric adenocarcinoma. This gene, which is coupled to transcription of a downstream methylase enzyme, therefore regulates an important restriction enzyme based protection system for the organism, which could potentially allow for more successful (long-term) colonization. It is possible that isolates from cancer patients are more likely to be *iceA1* because these patients tend to be older, and by inference, infected for a longer period of time. Sequencing suggested that the *iceA2* genotype could produce proteins that differed in secondary structure. This might alter the function of the gene product. Interestingly, the *iceA2C* variant, which is readily expressed, was found most often in patients without clinically significant disease. In contrast, the 2D variant, which has postulated transmembrane regions and is not readily transcribed, was a marker for patients with peptic ulcer disease. Half of the isolates in this study were both *iceA1* and *iceA2* positive. An important question would be to gauge which gene would have the dominant effect. One may speculate that the peptic ulcer disease isolates may be more *iceA1*-like than *iceA2* because 2D variant is not readily transcribed (at least *in vitro*). In contrast the gastritis alone isolates may be more *iceA2*-like, because the 2C variant has been shown to be readily transcribed, while there is some evidence for mutations in the *iceA1* gene which alters its transcription and therefore function.

In summation, a "virulent fingerprint" (*vacA* s1bm1/*cagA*$^+$/intact *cag*PAI, *iceA1*) identified 80-100% of isolates from patients with clinically significant disease. It is

noteworthy that isolates both from patients with peptic ulcer disease and with gastric adenocarcinoma tended to share a large proportion of "virulence" genes, with only a few exceptions (the length of the *cagA* 3'-fragment and the distribution of the *iceA* genotypes). The latter may contribute to the differences in disease presentation. Not all patients infected with "virulent" *H. pylori* organisms, however, developed a clinically significant gastroduodenal disease. It is possible that these studies are limited by timing; only those organisms present in the gastric milieu at the time of clinical investigation are analyzed. The results of the first part of this study can only demonstrate unequivocally that at a particular period in the natural history of infection, certain strains are more likely to be associated with pathology. Other factors (bacterial or host) must play a role in disease etiology. The possibility that some strains are non-virulent is difficult to prove but is suggested by the findings.

Geographic analyses of the organism intimate that there is some evidence for a specific African clone of *H. pylori*. Interestingly, the *cagA* gene in this study is "European" in origin. This suggests that the *cag*PAI in the Western Cape samples may be derived from a single ethnic source that then swept through the bacterial population. The widespread prevalence of this multigene locus (100% of strains) suggests that it may have engendered a survival advantage to the organism. This study has identified a clonal grouping associated with peptic ulcer disease that might possibly reflect a progenitor source for a peptic ulcer disease-causing organism in the Cape-colored population. The different contributions of virulence factors derived from "African", "European" and "Asian" sources are not known and still require to be carefully examined in our study population.

The hypothesis that lipopolysaccharides may express the virulence potential of *H. pylori* organisms was tested in the second part of this study. A toxigenic LPS modified both the secretion of a gastric mitogen (histamine), as well as the proliferation of the naïve and transformed neuroendocrine ECL cell *in vitro*. In addition, the gastric adenocarcinoma AGS cell line was stimulated both to enter the cell cycle as well as to synthesize DNA. These latter effects were differentially modulated by South African isolates with differences in virulence (long *cagA* 3' and intact *cag*PAI) and correlated to some extent with disease type (gastric cancer) The mechanism by which this occurred appeared to be via activation of CD14 and intracellular production of the IP_3 pathway. These studies were conducted *in vitro* and it is possible that the results are limited to these systems. Other effectors (surface

receptors/intracellular pathways) may also be involved. The effects of LPS have not been properly investigated in *in vivo* models and it remains a possibility that the *in vitro* results are not transferable to the intact host.

The data presented in these studies, however, are supportive of a role for *H. pylori* virulence factors in the etiology of clinically significant gastroduodenal diseases in the Western Cape and provides a mechanistic basis for how this potentially may occur (Figure 13.1).

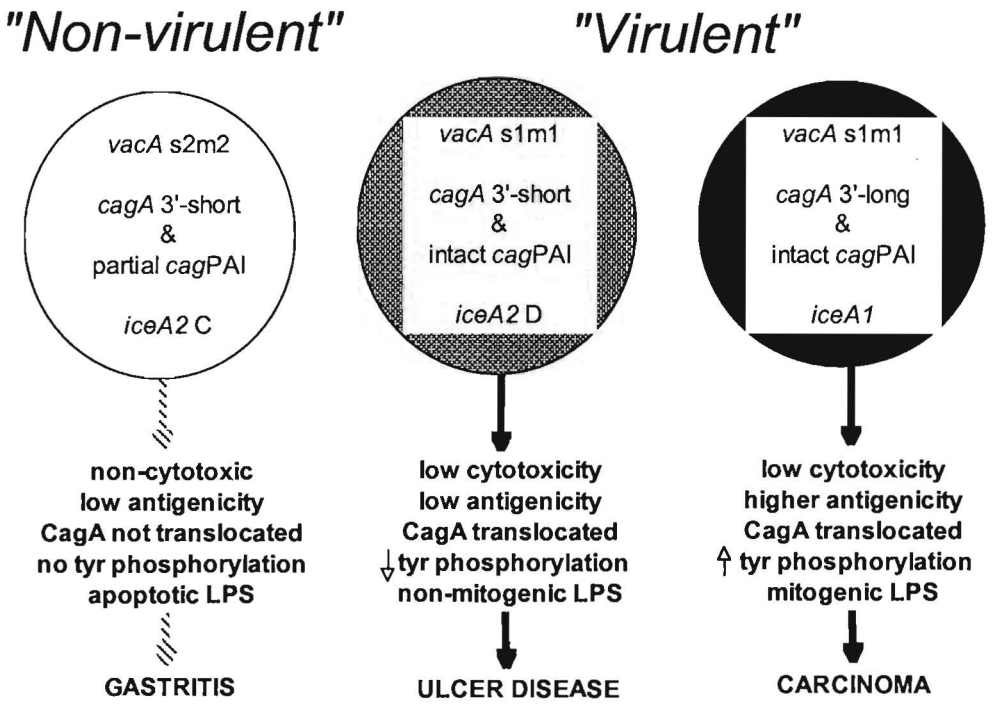


Figure 13.1 Relationship between virulence genes, potential *in vivo* effects and disease presentation – a hypothesis based on the study data. Other factors, e.g. host genetic polymorphisms may play a modulatory role.

Despite the widespread prevalence of virulence-associated genes and their specific DNA stimulatory effects *in vitro*, a significant number of infected patients do not develop clinically significant disease. Conversely, some patients with disease have alterations in some, but not all of the virulence genes. A number of important areas of research that may clarify these anomalies require to be undertaken in the future.

1) The mechanisms by which South African *H. pylori* strains affect epithelial cellular function need to be investigated. This would involve *in vitro* co-colonization studies, and

would determine whether isolates with differences in the *cag*PAI and in *cagA* or in the *iceA* genes differentially affect signal transduction pathways (specifically CagA tyrosine phosphorylation) in model cell systems.

2) The structure of LPS from differentially virulent organisms should be examined, in particular, whether the lipid A portion or the O-side chains of this complex chemical is the effector of DNA synthesis and cell proliferation/apoptosis. The mechanism by which this potentially may occur, either via activation of CD14 or intercalation into the cell membrane with subsequent modulation of putative protein signaling, require elucidation.
3) Various host factors should be investigated in infected patients. These could include a study of polymorphisms in the IL-1β gene (which have been associated with alterations in gastric acid secretion and are risk factors for gastric adenocarcinoma). Alterations in the balance of the Th1/Th2 immune response (which has been shown to modulate gastric colonization in a mouse model of *Helicobacter* infection) should also be examined. These, and other host phenomena, may potentially modulate the colonization and/or virulence of *H. pylori* and may shed some additional light on the natural history of infection with the organism in different populations.

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