Determinants of Vacuolating Cytotoxin Production and its Impact on *Helicobacter pylori* Pathogenesis

Charlotte Grace Masters, BSc (Hons)

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy.

July 2010

Declaration

Unless otherwise acknowledged, the work presented in this thesis is my own; no part has been submitted for another degree at the University of Nottingham or any other institute of learning.

Charlotte Masters.

September 2009.

Determinants of Vacuolating Cytotoxin Production and its Impact on *Helicobacter pylori* Pathogenesis

Introduction: The vacuolating cytotoxin is an important *H. pylori* virulence factor and is naturally polymorphic, one of the most diverse regions being the signal region (type s1 or s2). Signal type determines vacuolating activity: type s1 strains are vacuolating and are associated with peptic ulcers and gastric adenocarcinoma; and type s2 strains are non-vacuolating. Heterogeneity in VacA levels between strains also exists, and different strains produce different amounts: this is due to differences in *vacA* transcription and differences in protein secretion between strains. A *vacA* promoter and a transcriptional start point (TSP) have been identified. Sequence analysis revealed that -35 and -10 motifs are well conserved. Mutagenesis of this region resulted in a decrease in *vacA* transcription *in vitro*. However, investigation of *vacA* expression between 8 different *H. pylori* strains using real-time PCR revealed that differences in *vacA* transcription observed between different strains were unrelated to the putative -35 and -10 motifs. VacA type s1 and s2 signal sequences differ in the cleavage recognition site at key positions. In type s1 the more favourable serine and proline residues are at positions -3 and -6 respectively, whereas type s2 strains have leucine present at -3 and glycine at -6. In addition to VacA type, the determinants of VacA production are of importance in *H. pylori* pathogenesis: It has been shown that larger amounts of toxin produce greater damage in animal models. However there is limited information on the level of *vacA* expression *in vivo* and disease.

Aims: To analyse the *cysS – vacA* intergenic regions of a large number of strains to identify motifs and natural polymorphic differences that contribute to different *vacA* mRNA levels between strains *in vivo* and *in vitro* using sequencing and real-time PCR. Then to investigate these regions of interest using site directed mutagenesis. Next, to determine whether naturally occurring differences in the signal peptide cleavage recognition sites between type s1 and s2 strains affect the secreted VacA levels between the two strains using isogenic mutants and ELISA and Western blotting. Finally, to determine whether there is an association between the level of *vacA* mRNA *in vivo* and pre-cancerous changes, infection density and disease outcome in infected patients.

Results and Conclusions: Analysis of the *cysS – vacA* intergenic region revealed that differences between strains in the -35 and -10 regions were not significantly associated with levels of *vacA* transcription, but that other regions were associated with differences in *vacA* production between strains. These regions differed *in vitro* and *in vivo,* which may imply *in vivo* regulation of *vacA* transcription. Strains with a fully palindromic inverted repeat upstream of the -35 motif expressed more *vacA* than other strains *in vitro* (P=0.01). Analysis of *vacA* transcription from the same strains *in vivo* revealed that strains with an A within a second stem loop located in the 5' UTR of the *vacA* transcript expressed more *vacA* than those with a G at this position (P=0.006). Site directed mutagenesis confirmed that replacing the G with an A in an isogenic mutant strain resulted in an increase in *vacA* transcript levels. As found for other bacterial secreted proteins, these results show that positions -6 and -3 are important in the VacA signal sequence and may affect signal sequence processing efficiency. The level of *vacA* mRNA *in vivo* was significantly positively associated with the severity of chronic inflammation, neutrophilic activity, glandular atrophy and infection density in the infected patient. Differences in toxin production between *H. pylori* strains could help explain why only some infections result in disease.

Oral presentations

British Society of Gastroenterology Annual Meeting (2008).

Masters CG, Letley DP, Doherty NC, Atherton JC. (2008) The effect of natural polymorphisms upstream of the *Helicobacter pylori* vacuolating cytotoxin gene, *vacA*, on *vacA* transcription levels. Gut vol. 56 (suppl. 1): A23.

American Gastroenterological Association, Digestive diseases week (2008).

Masters CG, Letley DP, Doherty NC, Atherton JC. (2008) The effect of natural polymorphisms in the promoter of the *Helicobacter pylori* vacuolating cytotoxin gene, *vacA*, on *vacA* transcription levels. Gastroenterology vol. 134 (suppl. 1): A85.

Poster presentations

British Society of Gastroenterology Annual Meeting (2007).

Masters CG, Letley DP, Atherton JC. (2007) Natural polymorphism in the *Helicobacter pylori* vacuolating cytotoxin signal sequence affects toxin production. Gut vol. 56 (suppl. 11): A69.

Acknowledgements

I would like to thank Darren Letley, Dr Neil Doherty and Professor John Atherton for all of their advice and support over the last few years. I could not have hoped to find more helpful and supportive supervisors. I would also like to thank the other members of the Nottingham *Helicobacter pylori* group, without whom this project could not have been achieved.

I am also very grateful to Dr Abed Zaitoun, and the Department of Histopathology, University Hospital, Queen's Medical Centre, Nottingham for their preparation of histological samples and their help with analysis.

On a personal level I would like to thank Matthew Sinnett, Victoria Pitt and my friends at the Centre for Biomolecular Sciences for their support, keeping me going through the tough times and for making my PhD years so enjoyable.

Finally I would like to thank the University of Nottingham for providing the funding for this project.

Contents

CHAPTER 1 1-1

1 INTRODUCTION..... 1-2

1.1 Helicobacter pylori ..... Error! Bookmark not defined.

1.1.1 Introduction and history 1-2

1.1.2 Microbiology 1-2

1.2 *H. pylori* and disease 1-3

1.2.1 Epidemiology, sources of infection and transmission 1-3

1.2.2 Clinical aspects of *H. pylori* associated disease..... 1-4

1.2.2.1 Gastric MALT lymphoma 1-6

1.2.2.2 Gastro-oesophageal reflux disease (GERD). 1-6

1.2.3 Immune response to *H. pylori* colonisation 1-6

1.2.4 Factors determining the outcome of *H. pylori* infection..... 1-9

1.3 *H. pylori* virulence and colonisation factors..... 1-11

1.3.1 Acid resistance 1-11

1.3.2 Motility..... 1-11

1.3.3 Adherence and outer membrane proteins 1-11

1.3.4 LPS..... 1-12

1.3.5 CagA and the *cag* pathogenicity island (PAI) 1-13

1.3.6 Duodenal ulcer promoting gene (*dupA*) 1-14

1.3.7 Induced by contact with epithelium A (IceA)..... 1-15

1.3.8 Vacuolating cytotoxin VacA 1-15

1.3.8.1 Oligomeric structure of VacA 1-19

1.3.8.2 *vacA* polymorphism..... 1-22

1.3.8.3 Disease associations and animal models 1-24

1.3.8.4 Effect of VacA on epithelial cells 1-26

1.3.8.4.1 Alteration of permeability 1-26

1.3.8.4.2 Pore formation in the cell membrane 1-27

1.3.8.4.3 Vacuolation 1-27

1.3.8.4.4 Apoptosis 1-30

1.3.8.5 Effects of VacA on cells of the immune system 1-31

1.4 Regulation of gene expression 1-33

1.4.1 Regulation of transcription Initiation 1-33

1.4.1.1 Sigma factors 1-34

1.4.1.2 Transcription factors 1-37

1.4.2 Two-component systems 1-42

1.4.3 Transcriptional termination 1-44

1.4.3.1 Factor-independent termination 1-44

1.4.3.2 Factor-dependent termination 1-44

1.4.3.3 Attenuation and antitermination 1-45

1.4.3.4 mRNA stability 1-47

1.4.4 Translational regulation 1-48

1.4.5 Phase-variation 1-51

1.4.6 Protein secretion 1-52

1.5 Determinants of VacA production 1-56

1.5.1 Genetic determinants of *vacA* expression 1-56

1.5.2 Environmental determinants of *vacA* expression 1-58

1.5.3 Other genetic factors potentially affecting VacA levels 1-59

1.6 Aims and objectives 1-60

CHAPTER 2 1-61

2 MATERIALS AND METHODS 2-62

2.1 Media 2-62

2.1.1 Luria-Bertani medium 2-62

2.1.2 Luria-Bertani agar 2-62

2.1.3 Brain heart infusion medium 2-62

2.1.4 Sulphite free brucella broth 2-63

2.1.5 Iso-Sensitest medium 2-63

2.1.6 Terrific broth 2-63

2.1.7 Blood agar 2-63

2.1.8 Antibiotics 2-64

2.2 Culture conditions 2-64

2.2.1 *E. coli* culture on LB agar plates 2-64

2.2.2 *E. coli* liquid culture 2-64

2.2.3 *H. pylori* culture on blood agar plates 2-64

2.2.4 Isolation of clinical strains from gastric biopsies 2-65

2.2.5 *H. pylori* Broth cultures 2-65

2.2.6 Culture of *H. pylori* in tissue culture medium 2-66

2.2.7 Co-culture of *H. pylori* and AGS cells 2-66

2.3 Collection of gastric biopsies 2-67

2.4 Strains and plasmids 2-68

2.5 Isolation, manipulation and analysis of DNA 2-72

2.5.1 Preparation of *H. pylori* genomic DNA 2-72

2.5.2 Natural transformation of *H. pylori* 2-72

2.5.3 Plasmid DNA extraction 2-73

2.5.4 Polymerase chain reaction (PCR) 2-73

2.5.4.1 Primers 2-74

2.5.5 Analysis of DNA by agarose gel electrophoresis 2-75

2.5.6 Purification of DNA fragments 2-75

2.5.7 DNA sequencing and analysis 2-75

2.5.8 Site directed mutagenesis 2-76

2.5.9 Transformation of *E. coli* by electroporation 2-76

2.6 Isolation and Analysis of RNA 2-77

2.6.1 Preparation of RNA from bacterial cultures and AGS cell co-culture 2-77

2.6.2 Preparation of RNA from gastric biopsies 2-78

2.6.3 Quantitation of total RNA 2-79

2.6.4 DNA-free treatment of RNA 2-79

2.6.5 cDNA synthesis 2-80

2.6.6 Quantitative real-time PCR 2-80

2.7 Isolation and Analysis of Protein 2-82

2.7.1 Separation of secreted proteins by centrifugation 2-82

2.7.2 Preparation of cellular proteins 2-82

2.7.3 SDS-PAGE analyses of *H. pylori* proteins 2-82

2.7.4 Coomassie blue staining 2-83

2.7.5 Western blotting 2-83

2.7.6 Quantitation of immunoreactive protein concentrations 2-84

2.7.7 N-terminal protein sequencing 2-84

2.7.8 VacA enzyme linked immuno-sorbent assay (ELISA) 2-85

2.8 Histopathology 2-86

2.9 Statistical analysis 2-87

3 INVESTIGATION INTO ASSOCIATIONS BETWEEN LEVELS OF *VACA* TRANSCRIPTION *IN VIVO* AND DISEASE. 3-90

3.1 Introduction 3-90

3.1.1 Aims 3-94

3.2 Results 3-95

3.2.1 Quantitative real-time PCR showed large differences in *vacA* transcription between strains 3-95

3.2.2 *vacA* and *cagA* genotyping of the infecting strains 3-105

3.2.3 Relationships between *vacA* mRNA level and histological severity 3-107

3.2.4 *vacA* transcript level is associated with the severity of inflammation, neutrophilic activity and atrophy and the density of *H. pylori* infection 3-120

3.2.5 Association between *vacA* transcript levels and other histopathological parameters 3-125

3.2.6 Association with disease 3-125

3.3 Discussion 3-128

3.3.1 Limitations 3-134

3.3.1.1 Sample size 3-134

3.3.1.2 Real-time PCR 3-136

3.3.2 Future work 3-137

CHAPTER 4 3-139

4 CHARACTERISATION OF THE EFFECT OF NATURAL POLYMORPHISMS IN THE *CYSS – VACA* INTERGENIC REGION ON *VACA* TRANSCRIPTION LEVELS *IN VITRO* AND *IN VIVO* 4-140

4.1 Introduction 4-140

4.2 Aims **4-144**

4.3 Results **4-145**

4.3.1 Characterisation of the effect of natural polymorphisms in the *cysS – vacA* intergenic region of clinical *H. pylori* strains *in vivo*. 4-145

4.3.2 Characterisation of the effect of natural polymorphisms in the *cysS – vacA* intergenic region of clinical *H. pylori* strains *in vitro*. 4-155

4.3.3 Quantitative real-time PCR using RNA extracted from broth cultures. 4-155

4.3.4 Assessment of *vacA* transcript levels throughout the growth curves of *H. pylori* clinical strains 4-160

4.3.5 Characterisation of the effect of natural polymorphisms in the *cysS – vacA* intergenic region of clinical *H. pylori* strains *in vitro*. 4-164

4.3.6 The level of *vacA* mRNA *in vivo* does not correlate with the level of *vacA* mRNA *in vitro* 4-172

4.3.7 Transcriptional readthrough from *cysS* is not responsible for the *vacA* mRNA level of a *H. pylori* strain. 4-174

4.4 Discussion **4-177**

4.4.1 Conclusions 4-182

4.5 Future Work **4-183**

CHAPTER 5 ..... **4-185**

5 INVESTIGATION INTO THE EFFECT OF AN UPSTREAM INVERTED REPEAT AND A 5' STEM-LOOP STRUCTURE IN THE *VACA* PROMOTER REGION ON *VACA* MRNA LEVELS. **5-186**

5.1 Introduction **5-186**

5.1.1 Aims 5-191

5.2 Results 5-192

5.2.1 Characterisation of the effect of deletion of the 5' stem-loop on *vacA* mRNA levels 5-192

5.2.2 Effect of co-culture on *vacA* mRNA levels..... 5-196

5.2.3 *H. pylori* culture in F12 Ham medium was optimised for analysis of *vacA* transcript levels at early time points 5-199

5.2.4 Construction of the 5' UTR stem-loop mutant: 60190/SL GtoA 5-201

5.2.5 Analysis of *vacA* mRNA levels in wild-type and mutant strains 5-202

5.2.6 Disruption of the upstream inverted repeat has no effect on *vacA* mRNA levels. . 5-206

5.2.7 Search for transcription factor binding sites within the *cysS-vacA* intergenic region 5-210

5.3 Discussion 5-213

5.3.1 Conclusions 5-222

5.4 Future work 5-224

CHAPTER 6 5-226

6 CHARACTERISATION OF THE EFFECT OF POLYMORPHIC DIFFERENCES IN THE REGION ENCODING THE VACA SIGNAL CLEAVAGE RECOGNITION SITE ON VACA PRODUCTION. 6-227

6.1 Introduction 6-227

6.1.1 Aims 6-232

6.2 Results 6-232

6.2.1 The mutations made by site directed mutagenesis in the signal region of *Helicobacter pylori* strains, 60190 and Tx30a were confirmed by sequencing. 6-235

6.2.2 Mutagenesis of the signal peptide affects VacA production in *H. pylori* strain 60190. 6-238

6.2.3 Mutagenesis of the signal peptide reduces VacA production in *H. pylori* strain Tx30a. 6-241

6.2.4 The mutant constructs pA153::*cat* and pA153::*cat* G(-6)PL(-3)S were transformed into *H. pylori* strain 60190 and the level of VacA secreted assessed. 6-244

6.2.5 Cell pellets of wild type and mutagenised *H. pylori* strains 60190 and Tx30a were analysed by western blotting. 6-249

6.2.6 Could differences in the signal peptidase explain differences in signal peptide processing between strains 60190 and Tx30a 6-251

6.2.7 BLAST search for *lepB* in 26695..... 6-251

6.2.8 The LepB sequences of 60190 and Tx30a differ but not in the -1 and -3 cleavage recognition pockets. 6-251

6.2.9 The mutations made in the signal sequence of 60190 and Tx30a do not force cleavage to occur at another site 6-255

6.3 Discussion 6-256

6.4 Conclusions 6-260

6.4.1 Limitations 6-261

6.5 Future work 6-262

7 DISCUSSION 7-264

7.1 Summary of results and their significance 7-264

7.1.1 Genetic determinants of *vacA* production..... 7-264

7.1.2 Environmental determinants of *vacA* expression 7-267

7.1.3 Regulation of production at the level of protein secretion 7-268

7.1.4 VacA and disease 7-270

7.2 Conclusions 7-273

7.3 Future work 7-274

7.3.1 Genetic determinants of *vacA* production..... 7-274

7.3.2 VacA and disease 7-275

8 REFERENCES.....8-279

List of Figures and Tables

Figure 1.1 VacA polymorphism and function. 1-17

Figure 1.2 Image of the top surface of a soluble VacA oligomer which has a diameter of approximately 30nm. 1-21

Figure 1.3 Atomic force microscopy image of membrane associated VacA showing a lattice of pore-like structures. 1-21

1.4 HeLa cells left untreated (A) or treated with *H. pylori* strain 60190 (s1/m1) water extract (B). 1-29

Figure 1.5 Signal sequences of VacA type s1a, s1b and s2. 1-55

Figure 3.1Amplification plots produced by real-time PCR machines. 3-98

Figure 3.2 Standard curve and amplification efficiency of *vacA* (A) and 16S (B). 3-101

Figure 3.3 Melt curves of 16S and *vacA* PCR products synthesised from template extracted from a *H. pylori* positive biopsy (A) and a *H. pylori* negative biopsy (B). 3-102

Figure 3.4 shows 2% agarose gels containing PCR products from *vacA* and *cagA* typing PCRs. 3-106

Figure 3.5 Sydney scoring system, using the visual analogue scales. 3-110

Figure 3.6 Slide showing normal gastric mucosa, not colonized by *H. pylori*. Grade 0 inflammation, activity, atrophy, intestinal metaplasia and *H. pylori* density. Slide stained with haematoxylin and eosine stain (H&E); x 10 magnification. 3-111

Figure 3.7 Slide showing active chronic *H. pylori* gastritis. The gastric mucosa contains low numbers of lymphocytes and plasma cells (grade 1 inflammation), no signs of polymorphonuclear activity (grade 0 activity), atrophy (grade 0 atrophy) or intestinal metaplasia (grade 0 intestinal metaplasia). Slide stained with H&E; magnification x 20. 3-112

Figure 3.8 Slide showing moderate chronic antral gastritis with lymphoid follicle. The low power view shows moderate chronic inflammation (grade 2) and glandular atrophy (grade 2). Slide stained with H&E; magnification x10. 3-113

Figure 3.9 Slide showing activity in chronic *H. pylori* antral gastritis. Neutrophils are marked with an arrow. Slide stained with H&E; magnification, x 20. 3-114

Figure 3.10 Slide showing mild chronic antral *H. pylori* gastritis. The low power view shows mild inflammation (grade 1) with low grade atrophy (grade1). Slide stained with H&E; magnification x 10. 3-115

Figure 3.11 Slide showing antral gastritis with marked atrophy (grade 3). Slide stained with H&E; magnification, x 20. 3-116

Figure 3.12 Slide showing grade 2 *H. pylori* colonization density. *H. pylori* marked with an arrow. Slide stained with toludine blue; magnification, x 100. 3-116

Figure 3.13 Slide showing grade 2 *H. pylori* colonization density. *H. pylori* marked with an arrow. Slide stained with toludine blue; magnification, x 100. 3-117

Figure 3.14 Slide showing moderate chronic inflammation and atrophy (grade 2) and marked intestinal metaplasia (grade 3). Goblet cells (an indicator of the presence of intestinal metaplasia) are marked with arrows. Slide stained with (H&E); magnification, x 10. 3-118

Figure 3.15 Slide showing antral gastritis with mild intestinal metaplasia (grade 1). Goblet cells are marked with arrows. Slide stained with alcian blue/PAS; maginification, x 40. 3-118

Figure 3.16 Slide showing antral gastritis with marked intestinal metaplasia (grade 3). Goblet cells are marked with arrows. Slide stained with alcian blue/PAS; maginification, x 40. 3-119

Figure 3.17 Box and whisker plot showing the *vacA* mRNA level of strains in gastric biopsies taken from patients with mild, moderate and severe inflammation (A) and mild versus moderate and severe inflammation (B). 3-122

Figure 3.18 Box and whisker plot showing the *vacA* mRNA level of strains in gastric biopsies taken from patients with no, mild and moderate activity (A) and activity versus no activity (B). 3-123

Figure 3.19 Box and whisker plot showing the *vacA* mRNA level of strains in gastric biopsies taken from patients with atrophy and no atrophy. 3-124

Figure 3.20 Box and whisker plot showing the *vacA* mRNA level of strains in gastric biopsies taken from patients with grade 1 infection density and grade 2 or 3 infection density. 3-124

Figure 3.21 Box and whisker plot showing the *vacA* mRNA level in gastric biopsies taken from patients with disease and no disease. 3-127

Figure 4.1 Comparison of the *cysS* – *vacA* intergenic regions of clinical strains *in vivo*. 4-148

Figure 4.2 Standard curve and amplification efficiency of 16S (A) and *vacA* (B). To determine the amplification. 4-158

Figure 4.3 Melt curves of 16S and *vacA* PCR products synthesised from template extracted from broth culture. 4-159

Figure 4.4 Growth profiles of clinical *H. pylori* strains 73B, 173B, 201B and 221B. 4-162

Figure 4.5 Comparison of *vacA* mRNA levels at 15, 18 and 21 hours in clinical *H. pylori* strains. 4-163

Figure 4.6 Comparison of the *cysS* – *vacA* intergenic regions of clinical strains cultured *in vitro*. 4-165

Figure 4.7 *vacA* mRNA level *in vivo* does not correlate with *vacA* mRNA level *in vitro*. 4-173

Figure 4.8 *vacA* mRNA levels are not due to transcriptional readthrough from *cysS*. 4-176

Figure 5.1 The putative stem-loop begins at the +4 site of the 5' untranslated region of *vacA*. 5-190

Figure 5.2 Sequence Alignment of 60190/ΔSL. 5-194

Figure 5.3 *vacA* mRNA levels of strains 60190/pCTB2::*cat* and 60190/ΔSL grown for 18 hours in broth culture. 5-195

Figure 5.4 VacA Western blot of control strain 60190/pCTB2::*cat* and stem-loop deletion mutant 60190/ΔSL. 5-195

Figure 5.5 *vacA* mRNA levels of strains 60190/pCTB2::cat and 60190/ΔSL grown for 30 minutes, 3 hours and 22 hours in (A) co-culture with AGS cell and (B) in F12 Ham medium without AGS cells determined using quantitative real-time PCR. 5-198

Figure 5.6 *vacA* mRNA levels of strains 60190/pCTB2::cat and 60190/ΔSL grown for 20 minutes, 40 minutes and 180 minutes in 10ml F12 Ham medium determined using quantitative real-time PCR. 5-200

Figure 5.7 The stem-loop deletion mutant 60190/SL GtoA and the control strain 60190 pCTB2::cat were sequenced using primer A1043. 5-204

Figure 5.8 *vacA* mRNA levels of strains 60190/pCTB2::cat and 60190/ΔSL grown for 30 minutes and 180minutes in 10ml F12 Ham medium determined using quantitative real-time PCR. 5-205

Figure 5.9 The disrupted inverted repeat mutant 60190/IRDIS and the control strain 60190/pCTB2::cat were sequenced using primer A1043. 5-208

Figure 5.10 *vacA* mRNA levels of strains 60190/pCTB2::cat and 60190/IRDIS grown for 18 hours in broth culture determined using real-time PCR. 5-209

Figure 5.11 Direct repeats were observed directly upstream of the 63 bp deleted region. 5-212

Figure 6.1 Signal sequences of VacA type s1a, s1b and s2. 6-230

Figure 6.2 Sequence alignment of wild-type *H. pylori* strain 60190, and its isogenic VacA signal sequence mutant derivatives. 6-236

Figure 6.3 Sequence alignment of wild-type *H. pylori* strain Tx30a, and its isogenic VacA signal sequence mutant derivatives. 6-237

Figure 6.4 Mean VacA ELISA values for the control *H. pylori* strain 60190/pCTB2::cat and its isogenic VacA signal sequence mutant derivatives. 6-240

Figure 6.5 A representative VacA western blot of control strain 60190/pCTB2::cat and its isogenic VacA signal sequence mutant derivatives. 6-240

Figure 6.6 Mean VacA ELISA values for the control *H. pylori* strain Tx30a/pA153::cat and its isogenic VacA signal sequence mutant derivatives. 6-243

Figure 6.7 A representative VacA western blot of control strain Tx30a/pA153::cat and its isogenic VacA signal sequence mutant derivatives. 6-243

Figure 6.8 Sequence alignment of wild-type *H. pylori* strain 60190, and its isogenic VacA signal sequence mutant derivatives. 6-247

Figure 6.9 VacA western blots of strains 60190, 60190/pA153::cat and 60190/pA153::cat G(-6)PL(-3)S. 6-248

Figure 6.10 Bacterial cell adsorbed VacA levels of control strains and VacA signal sequence mutants.. 6-250

Figure 6.11 Diagram showing the location of the S3 and S1 binding pockets in relation to the -1 and -3 residues in the signal peptide cleavage recognition site. 6-253

Figure 6.12 Sequence alignment of the deduced amino acid sequences of *E. coli* Lep and the *H. pylori* Lep homologue in strains 26695 (s1), 60190 (s1) and Tx30a (s2). 254

List of Abbreviations

BSA	Bovine serum albumin
bp	Base pair
Da	Daltons
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbant Assay
FCS	Fetal calf Serum
h	Hours
IL	Interleukin
kb	Kilobase
LPS	Lipopolysaccharide
M	Molar
mA	Milliamperes
mg	Milligrams
mM	Millimolar
ml	Milliliters
mm	Millimeter
min	Minutes
NFW	Nuclease free water
ng	Nanograms
NOD	Nucleotide-binding oligomerisation domain
OD	Optical Density
PAMP	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PBS	Phosphate Buffered saline
PRR	Pattern recognition receptor
Real-time PCR	Real-time quantitative reverse transcription PCR
rpm	Rotations per minute
s	Seconds
SDS	Sodium Dodecyl Sulphate

SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
std dev	Standard deviation
TLR	Toll-like receptor
U	Units
V	Volts
v/v	volume to volume
w/v	Weight to volume
μg	Micrograms
μl	Microlitres
μM	Micromolar
ρM	Picomolar

Chapter 1

1 Introduction

1.1 Helicobacter pylori

1.1.1 Introduction and history

The presence of spiral shaped bacteria in the human stomach was first observed by Konjetzny in 1923. However proof for an infectious origin of diseases of the upper gastrointestinal tract was not provided until the early 1980s when Warren and Marshall isolated *H. pylori* from gastric biopsies and established the bacterium's association with gastritis and peptic ulceration (Marshall and Warren, 1984). Following this discovery a large amount of research has been carried out over the last 2 decades, leading to the development of diagnostic tests and antibiotic treatment strategies for *H. pylori* infection. However, despite this intense investigation, *H. pylori* colonisation is still widespread in developing countries. Its route of transmission is poorly understood and emerging antibiotic resistance has consequences for the efficacy of treatment. Research into *H. pylori* transmission and disease is therefore important in developing novel prevention and treatment strategies.

1.1.2 Microbiology

H. pylori is a microaerophilic Gram negative bacterium which is able to colonise and persist in the mucus layer of the human stomach (Atherton, 2006). Cells are between 0.5 and 5 µm in length and are usually curved or spiral shaped with 5 – 7 unipolar sheathed flagella of approximately 3 µm in length which allow rapid motility through the viscous mucus layer (Goodwin *et al*., 1985). Morphological changes to a viable but non culturable coccoid form

occur after prolonged culture or antibiotic treatment (Enroth *et al*., 1999, Kusters *et al*., 1997, Monstein and Jonasson, 2001).

Sequenced *H. pylori* genomes are approximately 1.7 Mbp in size and have a G+C content of 35-40% and contain two copies of the 16S, 23S and 5S rRNA genes (Tomb *et al*., 1997). *H. pylori* is genetically heterogeneous, which results in every infected individual carrying a distinct strain (Kansau *et al*., 1996) genetic heterogeneity is thought to occur mainly via DNA rearrangement and the introduction and deletion of foreign sequences (Falush *et al*., 2001, Achtman and Suerbaum, 2000). It is thought that generation of heterogeneity may be an adaptation of *H. pylori* to the immune response and the gastric environment (Kuipers and Meijer, 2000).

1.2 *H. pylori* and disease

1.2.1 Epidemiology, sources of infection and transmission

H. pylori colonises the gastric mucosa which leads to chronic active gastritis, and generally persists for the life-time of the infected individual unless eradicated. The prevalence of *H. pylori* varies greatly between developed and developing countries. In developing countries approximately 80% of the population are *H. pylori* positive, but the prevalence of infection is relatively constant. In developed countries *H. pylori* prevalence generally remains under 40% and is rapidly declining. The exact mechanisms of transmission of *H. pylori* are largely unknown, but transmission is thought to occur via direct human to human contact via either oral to oral or fecal to oral route or both.

Several studies by PCR have reported presence of *H. pylori* DNA in water, but this may be due to contamination of water with naked DNA or non-viable *H. pylori*.

1.2.2 Clinical aspects of *H. pylori* associated disease

Colonisation with *H. pylori* is a risk factor for development of peptic ulcer disease (Kuipers *et al*., 1995b) and distal gastric cancer (Parsonnet *et al*., 1991a, Parsonnet *et al*., 1991b). Infected individuals have a 10-20% greater risk of developing peptic ulcer disease and a 1-2% greater risk of developing distal gastric cancer during their lifetime (Ernst and Gold, 2000, Kuipers *et al*., 1999, Kuipers *et al*., 1995b, Kuipers *et al*., 1995d, Kuipers *et al*., 1995e, Kuipers *et al*., 1995f). Almost all individuals infected with *H. pylori* develop gastritis, however only a small proportion of infected individuals progress to symptomatic disease (Moss and Blaser, 2005).

The pattern of gastritis plays an important role in the disease outcome in *H. pylori* infection. Antral predominant gastritis usually occurs in individuals with increased acid secretion resulting in *H. pylori* colonisation predominantly in the antrum where few acid secreting parietal cells are present. Antral predominant gastritis is associated with duodenal ulcer disease and is also characterised by increased polymorphonuclear cell activity and active chronic inflammation and gastric metaplasia in the duodenum. Individuals with antral predominant gastritis have limited inflammation and low numbers of bacteria colonising the corpus. Pan-gastritis usually occurs in individuals with impaired

acid secretion who have a more even distribution of *H. pylori* cells, with bacteria present in both the antrum and the corpus. Reduction in acid secretion can be due to a loss of parietal cells as a result of atrophic gastritis and can also occur when parietal cell function is inhibited by PPIs (Kuipers *et al*., 1995a). Pangastritis is associated with gastric ulcer disease and gastric adenocarcinoma and is characterised by chronic inflammation, more rapid progression towards atrophy (Kuipers *et al*., 1996), and intestinal metaplasia.

Inflammation caused by chronic *H. pylori* infection can eventually lead to the loss of the normal gastric mucosal architecture, with destruction of gastric glands and replacement by fibrosis and intestinal type epithelium. Atrophic gastritis and intestinal metaplasia occurs in approximately half of the *H. pylori* positive population and increases the risk of gastric cancer by 5 to 10 fold depending on the extent and severity of atrophy (Sipponen *et al*., 1985). The risk for atrophic gastritis depends on the distribution and pattern of chronic active inflammation. Areas of gland loss and intestinal metaplasia extend with time, although they do not give rise to specific symptoms.

Two histologically distinct types of gastric adenocarcinoma exist: the diffuse type which is characterised by gastritis throughout the stomach but not necessarily with gastric atrophy and the intestinal type which is characterised by a corpal predominant gastritis with gastric atrophy and intestinal metaplasia. The intestinal type of adenocarcinoma progresses through a cascade of histological steps which are initiated by chronic superficial gastritis which persists in the host for life unless specifically treated. Whether chronic active

gastritis then leads to atrophic gastritis is in part dependent on the host genetics, immune response, diet and levels of gastrin. Intestinal metaplasia follows leading to dysplasia and gastric adenocarcinoma (Peek and Blaser, 2002, Sipponen and Correa, 2002, Correa, 1996, Correa *et al*., 1975).

1.2.2.1 Gastric MALT lymphoma

H. pylori infection is a risk factor for the development of gastric MALT lymphoma (Parsonnet and Isaacson, 2004, Parsonnet, 1994, Parsonnet *et al*., 1994). However MALT lymphoma is relatively rare with less that 1% of *H. pylori* positive individuals affected (Parsonnet and Isaacson, 2004).

1.2.2.2 Gastro-oesophageal reflux disease (GERD).

GERD was considered to occur independently of *H. pylori* colonisation. However, further studies have found that colonisation with *H. pylori* may protect against the development of GERD. This protective effect is thought to be due to the acid suppressive effect of *H. pylori* induced corpus predominant gastritis.

1.2.3 Immune response to *H. pylori* colonisation

H. pylori colonization activates both the innate and acquired immune responses. This immune response to *H. pylori* infection persists for life and plays a central role in pathogenesis. Recognition of microorganisms by the innate immune system are largely reliant on the recognition of semi conserved bacterial products or pathogen associated molecular patterns (PAMPs) both on the surface of the bacteria and within the products shed as a results of bacterial

lysis (Nurnberger *et al*., 2004). Pattern recognition receptors (PRRs) expressed by cells of the mucosal epithelium and immune system, recognise a wide range of PAMPs including peptidoglycan, LPS, CpG motifs and flagellar (Hornef and Bogdan, 2005). Two main families of PRRs are known to be involved in *H. pylori* infection, the Toll-like receptor family (TLR) and the family of intracellular nucleotide-binding oligomerisation domain (NOD) PRRs (Ferrero, 2005). Stimulation of these pattern recognition receptors results in NF-κB activation and pro-inflammatory gene expression (Jung *et al*., 1997, George *et al*., 2003).

H. pylori infection has been reported to stimulate pro-inflammatory gene expression via TLR2, TLR4, TLR5 and TLR9 (Torok *et al*., 2005, Ding *et al*., 2005, Ishihara *et al*., 2004, Gewirtz *et al*., 2004). However *H. pylori* has evolved mechanisms to avoid global activation of this system. For example TLR4, recognizes bacterial LPS, yet due to lipid A core modifications *H. pylori* LPS is relatively anergic compared to that of other gram negative bacteria (Backhed *et al*., 2003). Furthermore, TLR5 recognizes and responds to bacterial flagellins expressed by *E. coli* but is not stimulated by *H. pylori* flagellin (Smith *et al*., 2003).

The intracellular pattern recognition receptor NOD1 recognizes soluble components of bacterial peptidoglycan (Viala *et al*., 2004). Bacterial peptidoglycan is delivered to the epithelial cell cytoplasm via the type IV secretion system which is encoded by the *cag* pathogenicity island (*cag*PAI) in *cag*PAI+ *H. pylori* strains which leads to NF-κB activation in epithelial cells

and the expression of pro-inflammatory and anti-apoptotic genes such as IL-8 and cyclooxygenase-2 (Karin, 2006, Woo *et al*., 2002, Hirata *et al*., 2006, Kim *et al*., 2001, Viala *et al*., 2004, Naumann and Crabtree, 2004). NOD1 represents a crucial mechanism for the induction of *H. pylori* associated inflammation and its NF-κB activation via NOD1 is important for the induction of *H. pylori* associated inflammation and thus pathogenesis (Boughan *et al*., 2006).

The cytokines produced following PRR stimulation which include IL-6, IL-8, IL-10, IL-12, IL-1β and TNFα, are involved in the migration of granulocytes, monocytes and lymphocytes into the mucosa. High densities of infiltrating cells are associated with more severe inflammatory pathology (Ernst; *et al* 2000). The influx of new immune cells results, with their own array of PRRs which are also then exposed to bacterial components, which leads to further pro-inflammatory gene expression.

H. pylori also activates a specific acquired immune response including generation of antibodies and effector T cells (Bamford *et al*., 1998). Although this involves a Th1 and Th2 cell component, *H. pylori* infection in humans is Th1 poliarized (Bamford *et al.,* 1998). Th1 cells secrete IL-2 and IFN-γ and promote cell-mediated immune responses, whereas Th2 cells produce IL-4, IL-10, IL-5 and IL-6 and induce B-cell activation and differentiation. The majority of intracellular bacteria induce a Th1 response, whereas extracellular toxin producing bacteria usually stimulate a Th2 response. However studies in mouse models have shown that a Th1 response results in a reduced *H. pylori*

colonization density suggesting that a Th1 response is appropriate to control *H. pylori* infection (Mohammadi *et al*., 1997).

A Th1 predominant response is a major factor in *H. pylori* pathogenesis: mice with a Th1 predominant response show more severe gastric inflammation than those with a Th2 predominant response (Mohammadi *et al.,* 1997) (Smythies *et al*., 2000). T cell transfer experiments in a mouse model have shown that these effects are dependent on Th1 cells (Mohammadi *et al.,* 1997). Furthermore it has been shown that IFNγ secreted by Th1 cells is key in the development of *H. pylori* associated disease; the number of IFNγ producing cells in the infected human gastric mucosa has been shown to correlate with the severity of gastritis (Lehmann *et al*., 2002) and it was found that mice infused with IFNγ developed pre-cancerous gastric atrophy, metaplasia and dysplasia even in the absence of infection (Garhart *et al*., 2002).

1.2.4 Factors determining the outcome of *H. pylori* infection

Although almost all individuals infected with *H. pylori* develop gastritis only a small minority go on to develop gastric cancer and several other risk factors are important in determining disease outcome. These include host genetics and inflammatory response, environmental factors such as diet and smoking and bacterial strain specific virulence factors which are discussed below.

Host genetic polymorphisms can affect the level of expression of IL-1 and other cytokines affecting the immune response to *H. pylori* infection. It has

been shown that individuals with proinflammatory genotypes have a higher risk of corpus predominant pangastritis which predisposes them to atrophic gastritis, intestinal metaplasia and gastric cancer (El-Omar *et al*., 2000).

Environmental factors, such as diet and smoking play an important part in determining the course of *H. pylori* associated disease. Smoking has been shown to be associated with an increased risk of peptic ulcer disease (Shimoyama *et al*., 2001, Atherton, 2006). Individuals whose diets are high in red meat and salt have been shown to be at an increased risk of developing gastric cancer (Atherton, 2006). As with other cancers, a diet rich in fruit, vegetables and cereals has been suggested to have mild to moderate prophylactic potential for gastric cancer although the specific food components acting as protective factors for gastric cancer still remain to be fully defined (Rocco and Nardone, 2007).

H. pylori uses an extensive repertoire of virulence and colonisation factors to overcome host defence mechanisms. These factors have been shown to play an important role in determining *H. pylori* pathogenesis. *H. pylori* colonisation and virulence factors and their role in *H. pylori* associated disease are discussed in detail below.

1.3 *H. pylori* virulence and colonisation factors

1.3.1 Acid resistance

Urease is essential for the survival of *H. pylori* in the acidic gastric environment (Eaton and Krakowka, 1994, Tsuda *et al*., 1994, Andrutis *et al*., 1995). It allows *H. pylori* to maintain a constant periplasmic and internal pH which is required for transmembrane potential difference. Urease is produced by all *H. pylori* strains although the level of urease activity differs significantly between *H. pylori* isolates (Contreras *et al*., 2003). Urease has been proposed to participate in tissue damage by producing ammonia which is thought to be cytotoxic to epithelial cells (Smoot *et al*., 1990).

1.3.2 Motility

Motility is essential for *H. pylori* colonization (Eaton *et al*., 1996, Ottemann and Lowenthal, 2002). Flagellar motility is thought to be required for the initial stages of infection allowing the bacterium to move from the acidic environment of the gastric lumen into the less acidic mucus layer (Schreiber *et al*., 2004). Mutants defective in the synthesis in either one of the two flagellins have impaired colonization efficiency. Non-motile double mutants are completely avirulent (Eaton *et al*., 1996).

1.3.3 Adherence and outer membrane proteins

Adherence to the gastric mucosa is thought to be important in the initial colonization and the long term persistence of *H. pylori* in the gastric mucosa. Five major outer membrane families that act as adhesins have been identified

and include the blood group antigen adhesin (BabA), sialic acid binding adhesion (SabA), outer inflammatory protein (OipA), adherence–associated lipoprotein (AlpAB) and HopZ. BabA recognises Lewis b antigen and related fucosylated ABO blood group antigens (Ilver *et al*., 1998) and mediates its attachment to surface mucus cells and cells in the gastric pit (Falk, 1995). It is thought to be the primary adhesin for human colonisation. *babA2*, the gene that encodes BabA is only present in some strains, but those that possess *babA2* are more common in patients with peptic ulcer disease or gastric cancer. BabA has been shown to be associated with increased gastric inflammation and may play a role in pathogenesis by promoting colonisation and inflammation (Petersson *et al*., 2006). SabA recognises sialyl-Lewis x and sialyl-Lewis a antigens (Mahdavi *et al*., 2002) and has been shown to be associated with increased gastric inflammation (Petersson *et al*., 2006). OipA is a 34 kD outer membrane protein which has been reported to promote gastric inflammation by inducing IL-8 production in gastric epithelial cells (Yamaoka *et al*., 2002). OipA producing strains are associated with duodenal ulceration (Yamaoka *et al*., 2002) and gastric cancer (Yamaoka *et al*., 2005).

1.3.4 LPS

Like other Gram negative bacteria, the outer membrane of *H. pylori* contains lipopolysaccharides (LPS) which are composed of a core oligosaccharide, a lipid moiety (lipid A) and an O-chain polysaccharide. The O-chain polysaccharide of the majority of *H. pylori* strains mimic Lewis and other human blood group antigens. These bacterial Lewis antigens display antigenic

variation and are thought to contribute to immune evasion by *H. pylori* (Appelmelk *et al*., 1997, Monteiro *et al*., 1998). Lewis antigens appear to play a limited role in adhesion (Mahdavi *et al*., 2003) and colonisation (Takata *et al*., 2002).

1.3.5 CagA and the *cag* pathogenicity island (PAI)

An important *H. pylori* virulence factor is the cytotoxin associated gene *cagA*. The CagA protein is encoded by the *cagA* gene, which is located within the cag pathogenicity island (*cag* PAI). The *cag* PAI encodes a type IV secretion system that facilitates CagA translocation into epithelial cells (Bourzac and Guillemin, 2005). The *cag* pathogenicity island (*cag* PAI) is present in approximately 60% of Western strains (Tomb *et al*., 1997, Alm *et al*., 1999, Censini *et al*., 1996, Akopyants *et al*., 1998). Nearly all East Asian *H. pylori* strains contain the *cag* PAI (van Doorn *et al*., 1999, Maeda *et al*., 1998, Maeda *et al*., 1999).

Although all *H. pylori* strains induce gastritis to varying degrees, *cagA* positive strains are significantly associated with an increased risk of more severe gastritis, atrophic gastritis, peptic ulcer disease and distal gastric adenocarcinoma (Crabtree *et al*., 1993, Crabtree *et al*., 1991, Peek *et al*., 1995, Kuipers *et al*., 1995c, Beales *et al*., 1996, Blaser, 1995, Parsonnet *et al*., 1997, Rudi *et al*., 1997, Queiroz *et al*., 1998).

Upon translocation into the epithelial, cell CagA becomes phosphorylated at tyrosine residues within specific phosphorylation motifs by the Src family of kinases (Selbach *et al*., 2002, Stein *et al*., 2002). Phosphorylated CagA activates the eukaryotic SHP-2 phosphatase that stimulates cytoskeletal changes, abnormal cell proliferation and cell signalling pathways (Tsutsumi *et al*., 2003, Higashi *et al*., 2004).

The number of CagA tyrosine phosphorylation motifs have been found to vary between strains. Both Eastern and Western strains have A and B phosphorylation motifs, Western strains have additional C motifs, which vary in number between different *H. pylori* isolates (Akopyants *et al*., 1998, Arents *et al*., 2001, Argent *et al*., 2007). In contrast East Asian strains do not possess C motifs but do possess a D motif. CagA proteins from East Asian strains possess stronger SHP-2 binding and cell scattering activities than CagA proteins from Western strains. There is a significant positive association between the numbers of type C phosphorylation motifs and gastric carcinogenesis in Western strains (Azuma *et al*., 2002).

1.3.6 Duodenal ulcer promoting gene (*dupA*)

The recently identified *dupA* gene encodes a VirB4 ATPase homologue which comprises both jhp0917 and jhp0918 (Lu *et al*., 2005). DupA has been found to increase IL-8 expression in epithelial cells (Lu *et al*., 2005). The presence of *dupA* in an infecting strain is associated with duodenal ulceration and increased mucosal neutrophil infiltration but appears to be a protective factor against

gastric cancer in some, but not all studies (Lu *et al.*, 2005; Argent *et al.*, 2007; Arachchi *et al.*, 2007).

1.3.7 Induced by contact with epithelium A (IceA)

IceA was highlighted by the isolation of mRNA transcripts from strains associated with PUD (Peek *et al*., 1998). Two polymorphisms were identified: *iceA1* for which transcription is up regulated following contact with epithelial cells and the inactive *iceA2* which is not. The biological significance of these genes is unclear; *iceA1* has been associated with peptic ulcer disease, but this association is not universal (Yamaoka *et al*., 2002).

1.3.8 Vacuolating cytotoxin VacA

In 1988, Leunk and colleagues discovered that cell free supernatants from broth cultures of *H. pylori* induced vacuolar degeneration of various cultured epithelial cell lines (Leunk *et al*., 1988). This effect was subsequently shown to be caused by a secreted protein toxin, designated VacA (Cover *et al*., 1992). *vacA*, the gene encoding the vacuolating cytotoxin was cloned from several strains of *H. pylori*. In each it has been estimated that the gene encodes a protein of 139-140 kDa (Telford *et al*., 1994b, Phadnis *et al*., 1994, Schmitt and Haas, 1994, Cover *et al*., 1994). VacA is translated into a pre-protoxin of approximately 1200 amino acids. The pre-pro toxin is composed of a 33 amino acid N-terminal signal peptide, a central passenger domain and a 50 kDa C-terminal domain. The N-terminal signal peptide and the C-terminal domain are both cleaved during secretion of the toxin from the cell. Once secreted the

central passenger domain forms the mature toxin and consists of the p37 subunit which is associated with the toxicity of VacA, and the larger p58 subunit which mediates toxin-epithelial cell binding. The p37 and p58 subunits are linked by a protease sensitive surface exposed hydrophilic loop region (Figure 1.1.). Both the p37 and the p58 subunits are required for toxin activity (Ye *et al*., 1999).

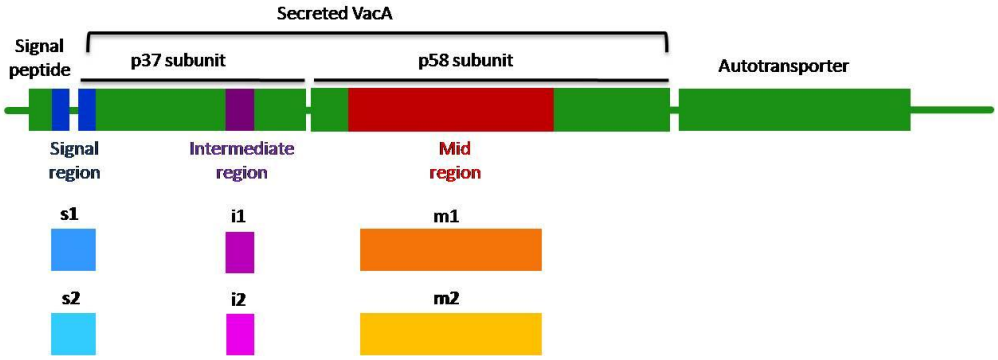


Figure 1.1 VacA polymorphism and function. VacA is polymorphic with two possible signal regions, s1 and s2, two possible intermediate regions i1 and i2 and two possible mid-regions, m1 and m2. The translated protein is an autotransporter with N- and C-terminal processing during bacterial secretion. The s1 signal region is fully active, but the s2 region encodes a protein with a different signal-peptide cleavage site, resulting in a short N-terminal extension to the mature toxin that blocks vacuolating activity and attenuates pore-forming activity. The mid-region encodes a cell-binding site, but the m2 type binds to and vacuolates fewer cell lines *in vitro*. (Adapted from Rhead *et al*., 2007).

The *vacA* gene encodes a protoxin approximately 140kDa in mass. VacA possesses features typical of an autotransporter protein including a 33 amino acid N-terminal signal sequence, a ca.90kDa N-terminal passenger domain, and a 33kDa C-terminal β domain (Phadnis *et al*., 1994). Autotransporters are a distinct family of secreted proteins which contain all the necessary elements for translocation across the outer membrane within their own polypeptide sequences (Henderson *et al*., 1998). The N-terminal signal sequence directs secretion via the sec apparatus and is cleaved at the inner membrane by a signal peptidase releasing the remaining portion of the VacA protoxin into the periplasm. It is unclear what happens in the periplasmic space, but it is thought that the protein may exist as an intermediate before the final stage of export. The amphipathic C-terminal domain forms a β barrel which inserts into the outer membrane to form a pore through which the rest of the protein passes. The C-terminal sequence is also cleaved by an unknown protease and the mature protein of approximately 90-95kDa is released as a soluble protein into the extracellular milieu (Reyrat *et al*., 1999; Schmitt *et al*., 1994; Henderson *et al*., 1998). An alternative model of autotransport secretion has been proposed by Veiga *et al.*, 2004 (Veiga *et al*., 2004) who suggest that a minimum of six β domains assemble as a ring shaped oligomeric complex in the bacterial outer membrane. The protein is translocated from the periplasm to the external medium by passing through the central hydrophilic pore formed by an autotransporter oligomeric complex. VacA may remain localised on the surface of the bacterium in toxin-rich patches (Ilver *et al*., 2004). These VacA rich patches can be transferred directly from the surface of *H. pylori* to the host

cells upon direct contact (Ilver *et al*., 2004). VacA has also been shown to be released from the bacterial cell in the form of membrane blebs (Fiocca *et al*., 1999, Keenan *et al*., 2000, Keenan and Allardyce, 2000).

1.3.8.1 Oligomeric structure of VacA

Once secreted into the extracellular environment, VacA exists as a 90-95kDa molecule that is composed of two domains separated by a surface exposed hydrophilic loop which is sensitive to proteolytic cleavage (Telford *et al*., 1994b). Cleavage in this region yields two subunits, p37 and p58 (named according to their molecular weight, sometimes also designated p33 and p55) that remain non-covalently associated. Using deep etch electron microscopy the p37 and p58 subunits have been shown to aggregate into large flower shaped or flat form complexes. The round form flower shaped complexes are approximately 30nm in diameter and appear to consist of a central ring surrounded by six or seven "petals" (Lupetti *et al*., 1996). The flat form complexes also consist of six or seven petals which radiate from the centre of the complex with a distinctive clockwise chirality but with no central ring (Figure 1.2.). In another model, the flower-like forms are suggested to be dodecamers or tetradecamers of VacA monomers of approximately 90kDa, and flat forms are suggested to be hexamers or heptamers (Cover *et al*., 1997). Atomic force microscopy imaging of purified VacA supports the deep-etch electron microscopy data and suggests that VacA is arranged in hexagonal central rings attached by connectors to peripheral domains (Figure 1.3.) (Czajkowsky *et al*., 1999).

Mutant forms of VacA that are unable to form oligomers also lack vacuolating activity (Vinion-Dubiel *et al*., 1999), which suggests that oligomerization is an important feature of purified VacA. Following exposure to acidic or alkaline conditions, these oligomeric forms become activated by dissociation into monomers (Cover *et al*., 1997, Molinari *et al*., 1998a, Yahiro *et al*., 1999), and thus results in enhanced internalisation of the toxin and a marked increase in its cytotoxin activity (Cover *et al*., 1997, de Bernard *et al*., 2000, McClain *et al*., 2000, Molinari *et al*., 1998a, Yahiro *et al*., 1999). However, culture supernatants and VacA associated with the bacteria do not seem to require activation (Leunk *et al*., 1988, Reyrat *et al*., 1999), suggesting that active VacA may be monomeric *in vivo*, and oligomerization, which requires acid activation for its function, might be an artefact of protein purification (Gebert *et al*., 2004).

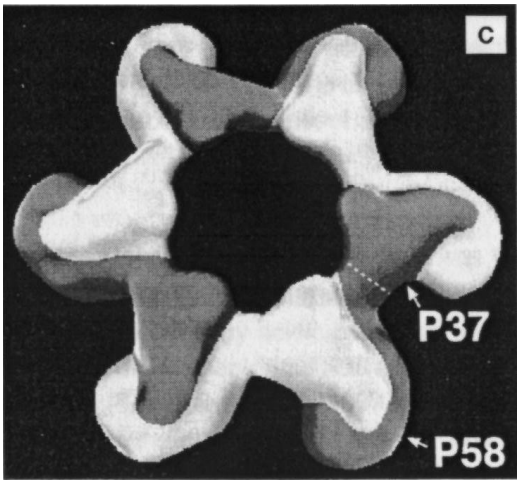


Figure 1.2 Image of the top surface of a soluble VacA oligomer which has a diameter of approximately 30nm. This is a three dimensional reconstruction from electron micrographs of quick-freeze deep-etch metal replicas of VacA. (Taken from (Lanzavecchia *et al.*, 1998)).

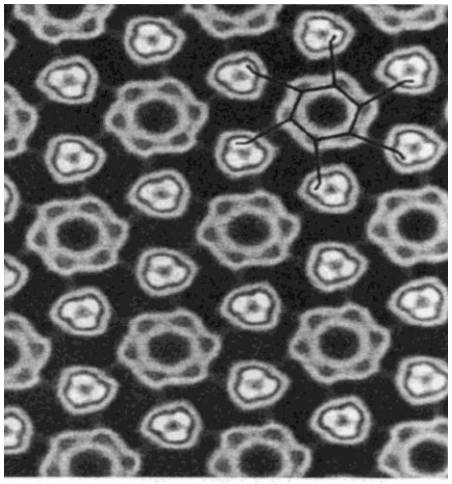


Figure 1.3 Atomic force microscopy image of membrane associated VacA showing a lattice of pore-like structures. The line diagram in the top right hand corner shows a VacA hexamer with an overall diameter of 28nm. (Taken from (Czajkowsky *et al.*, 1999).)

1.3.8.2 *vacA* polymorphism

vacA is present in all strains of *H. pylori* and is transcribed in a monocistronic form (Cover *et al*., 1994, Phadnis *et al*., 1994, Schmitt and Haas, 1994, Telford *et al*., 1994b). The gene is generally well conserved between strains but there is significant diversity in three distinct regions of the gene: the signal (s) region and the mid (m) region and the recently identified intermediate (i) region (Atherton *et al*., 1995). Characterisation of these regions has revealed that allelic variation exists (Atherton *et al*., 1995, van Doorn *et al*., 1998). Allelic diversity in *vacA* has allowed strains to be genotyped s1 or s2 for the signal region and m1 or m2 for the mid region and i1 or i2 for the intermediate region (van Doorn *et al*., 1998, Atherton *et al*., 1995). The *vacA* genotype is an important determinant of toxicity.

Among the type s1 strains, subtypes s1a, s1b and s1c have been identified; although type s1c is restricted to Asia. Type s2 VacA is cleaved at a different site in the signal peptide sequence to type s1 resulting in a form of VacA which has a 12 amino acid hydrophilic N-terminal extension whereas mature type s1 VacA has a hydrophobic N-terminus (Atherton *et al*., 1995, Letley and Atherton, 2000). This 12 residue extension to VacA has been shown to be responsible for the loss of vacuolation induction by s2 strains. This was further demonstrated by the addition of the 12 amino acid extension to type s1 VacA resulted in vacuolating cytotoxin that could no longer vacuolate eukaryotic cells (Letley and Atherton, 2000, Letley *et al*., 2003, McClain *et al*., 2001)

Conversely, removing the s2 extension from type s2 VacA to make the strain s1-like confers vacuolating activity (Letley *et al*., 2003).

The *vacA* mid region which encodes part of the p58 region has been shown to be associated with cell specificity (Atherton *et al*., 1995, Pagliaccia *et al*., 1998, Letley *et al*., 2003, Ji *et al*., 2000). m1 forms vacuolate a variety of cell types but m2 forms are more limited in the cells they vacuolate (e.g. RK13 and primary gastric cells.). Within the m-region, two main types (m1 and m2) have been identified (Atherton *et al*., 1995). Among East Asian isolates, a novel m2 subtype (m2b) was designated to distinguish it from the original m2 subtype, which is now designated m2a (van Doorn *et al*., 1998). Subtype m2b was only observed in strains that were of signal region type s1c.

The recently identified intermediate region can also be divided into two types (i1 and i2) (Rhead *et al*., 2007). This region is located between the signal and mid regions of *vacA*. Rhead *et al.*, (2007) observed that *vacA* s1/m1 type strains were nearly always type-i1 and that type s2/m2 strains were always type-i2. The s1/m2 strains varied in their intermediate region type however, they found that strains that were s1/i1/m2 had vacuolating activity whereas type s1/i2/m2 strains were non-vacuolating. Using mutagenesis experiments they confirmed that the i region type determines vacuolating activity in s1/m2 strains and that the i region influenced cell specificity in s1/m1 strains. Thus, the i region is thought to be involved in cytotoxin binding (Rhead *et al*., 2007).

The *vacA* gene may comprise any combination of signal intermediate and mid region types. Most of the *vacA* combinations have been detected in strains isolated from infected individuals, although the s2/m1 form of *vacA* is rarely found. The reasons why allelic variants of *vacA* persist are not fully understood, but the natural persistence of distinct polymorphic forms of *vacA* in diverse human populations implies that each may offer differing selective advantages.

1.3.8.3 Disease associations and animal models

The clinical significance of VacA has been assessed from studies in animal models, and from several observations in humans infected with *H. pylori*. Firstly, large quantities of purified VacA can induce ulcer-like erosions when administered into the mouse stomach (Marchetti *et al*., 1995). In addition to this, VacA increases the risk of gastric ulcer formation in experimentally infected gerbils (Ogura *et al*., 2000) and also enhances the bacterial colonization rate in a mouse model of infection (Salama *et al*., 2001). Orally administered toxigenic *H. pylori* sonicates and purified VacA also induce epithelial vacuolation, loss of gastric gland architecture (Ghiara *et al*., 1995, Telford *et al*., 1994a, Supajatura *et al*., 2002) and infiltration of mononuclear cells into the lamina propria (Telford *et al*., 1994a, Supajatura *et al*., 2002).

Several studies have investigated the association of *vacA* type and disease outcome. In Western populations such as the USA and Western Europe where *vacA* allelic diversity is common, *vacA* s1 genotypes are more frequently

associated with higher levels of inflammation in the gastric mucosa than s2 types (Atherton *et al*., 1995, Atherton *et al*., 1997, Evans *et al*., 1998, Kidd *et al*., 1999, van Doorn *et al*., 1998). *vacA* s1/m1 and s1/m2 strains have been shown to be associated with peptic ulceration and s1/m1 type strains have been associated with gastric carcinoma (Atherton *et al*., 1995, Atherton *et al*., 1997, Rhead *et al*., 2007, Basso *et al*., 2008). Corpus neutrophil infiltration was found to be more severe in patients infected with an m1 type strain than those infected with an m2 type strain (Umit *et al*., 2009). The *vacA* i1 type is a risk factor for peptic ulcer disease and has been shown to be associated with duodenal ulcer disease as well as gastric cancer (Basso *et al*., 2008, Rhead *et al*., 2007). Importantly Rhead *et al.*, 2007 found that although *vacA* s1, i1 and m1 alleles, along with *cagA* positive status were all associated with gastric adenocarcinoma in an Iranian population, the association was most pronounced with i1-type strains. Furthermore, only the i region status was independent of all other alleles studied. They concluded that the *vacA* i region is the best independent marker of toxicity and pathogenicity of a strain, and that the i1 i region type may be sufficient for identifying all pathogenic forms of *vacA*.

In East Asian and Southeast Asian countries the association between *vacA* allelic diversity and clinical outcome is not well established as the *vacA* allelic type is quite homogeneous with the majority of the population being infected with type s1/i1 strains (Maeda *et al*., 1998, Yamaoka *et al*., 1998). However, it is possible that a high prevalence of the more virulent *vacA* s1/i1 type strains may contribute to the high incidence of gastric cancer in this region of the world (Rhead *et al*,. 2007).

1.3.8.4 Effect of VacA on epithelial cells

In the past, VacA has mainly been regarded as a cytotoxin of the gastric epithelial cell layer. However, in addition to its well known vacuolating activity, VacA has been reported to induce apoptosis in epithelial cells (Cover *et al*., 2003; Galmiche *et al*., 2000; Kuck *et al*., 2001), to affect B lymphocyte antigen presentation (Molinari *et al*., 1998b), to inhibit the activation and proliferation of T lymphocytes (Fan *et al*., 1994; Shirai *et al*., 1998), and to modulate the T cell mediated cytokine response (Gebert *et al*., 2003), all of which may contribute to *H. pylori* persistence in the gastric niche.

1.3.8.4.1 Alteration of permeability

VacA has been shown to alter trans-epithelial resistance (TER). This appears to be due to modulation of the resistance of cell-cell junctions (Papini *et al*., 1998). This effect appears to be independent of vacuolation as the reduction in TER following VacA treatment is not accompanied by vacuolation, and TER decrease is not influenced by weak bases, which do affect vacuolation. Furthermore it is only partially affected by inhibitors of the VacA anion channel (Szabo *et al*., 1999). In addition to ions, small neutral molecules such as mannitol and sucrose also exhibit increased trans-epithelial diffusion upon VacA treatment. This has led to the suggestion that VacA is involved in the release of essential nutrients necessary for the growth of *H. pylori* in the gastric mucosa (Papini *et al*., 1998).

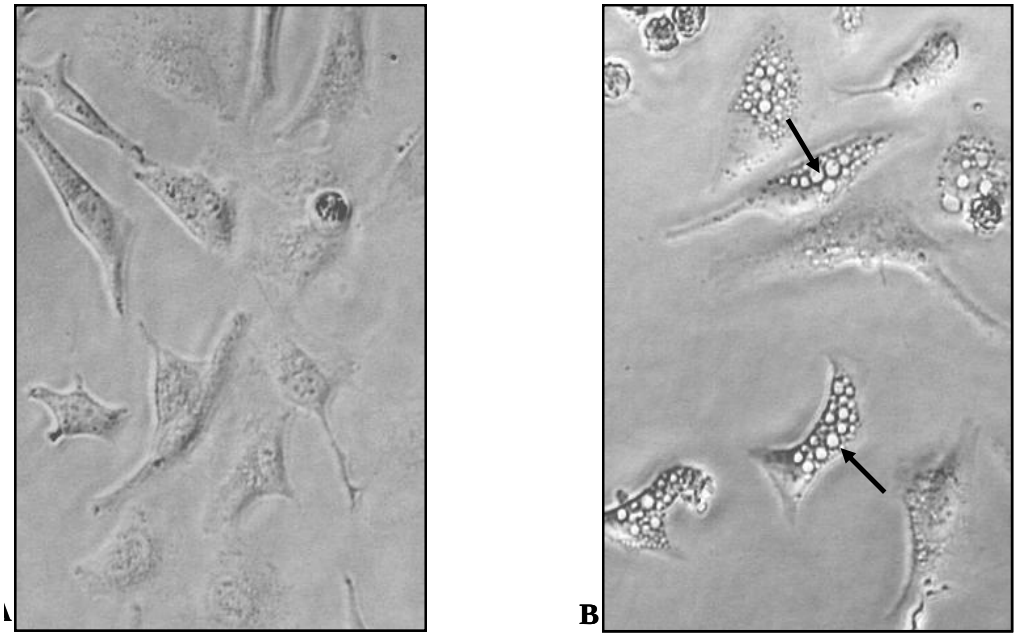
1.3.8.4.2 Pore formation in the cell membrane

VacA has been shown to form pores in epithelial cell membranes, allowing egress of anions and urea (Iwamoto *et al*., 1999, Tombola *et al*., 2001, Szabo *et al*., 1999, Tombola *et al*., 1999). This is important since urea hydrolysis catalyzed by *H. pylori* urease protects against gastric acidity allowing the bacterium to survive within the acidic environment of the stomach (Scott *et al*., 1998). Ammonia and bicarbonate, products of urea hydrolysis are also metabolic substrates for *H. pylori* (Burns *et al*., 1995).

1.3.8.4.3 Vacuolation

Vacuolation is the most apparent effect of VacA (Figure 1.4). It has been shown by McClain *et al*., (2000) that acid activation of purified VacA is required prior to internalisation, after which VacA localises in late endosomal vesicles and causes their expansion (Ricci *et al*., 1997). Vacuolation also requires vacuolar-ATPase and the presence of permanent weak bases, such as ammonia in the extracellular medium (Cover *et al*., 1993, Papini *et al*., 1993a, Papini *et al*., 1993b, Cover *et al*., 1992, Ricci *et al*., 1997). The requirement of vacuolar ATPase activity suggests that the intralumenal pH in vacuole precursors must be acidic in order for VacA to induce cell vacuolation. It seems likely that ammonium ions accumulate within the acidic lumen of these compartments, and cause their osmotically driven swelling (Papini *et al*., 2001). In addition to these, the cellular enzymes Rab7 (Papini *et al*., 1997), Rac1 (Hotchin *et al*., 2000) and dynamine (a high molecular mass G protein involved in the early stages of vesicle creation) are also required for vacuole

development and maintenance. The membrane trafficking protein Rab7 is a small GTPase, that is thought to be required to support membrane deposition and homotypic fusion between late endosomes, thus causing vacuole formation (Papini *et al*., 1997, Papini *et al*., 1994, Molinari *et al*., 1997). Rac1 is another GTP binding protein that is involved in the regulation of actin cytoskeleton rearrangements and intracellular signal transduction pathways (Hotchin *et al*., 2000). Both Rab7 and Rac1 have been shown to be essential for vacuolation as dominant negative mutants of Rab7 and Rac1 inhibit vacuolation.



HeLa - untreated HeLa treated with 60190

1.4 HeLa cells left untreated (A) or treated with *H. pylori* strain 60190 (s1/m1) water extract (B). Extensive vacuolation is observed in treated cells, highlighted by arrows. (Taken from (Narayanan, 2005).)

1.3.8.4.4 Apoptosis

Several studies have shown a correlation between *H. pylori* infection and an increased level of apoptosis in the human gastric mucosa (Moss *et al*., 1996, Mannick *et al*., 1996, Rudi *et al*., 1998). It has been shown by several groups that VacA induces apoptosis via a mitochondrial dependent pathway, which leads to cytochrome c release, and subsequent activation of the caspase-3-dependent cell death signalling cascade (Galmiche *et al*., 2000, Willhite and Blanke, 2004).

Investigation into the mechanism of VacA mediated cytochrome c release has revealed that VacA intoxication of cells leads to a breakdown of the mitochondrial transmembrane potential (Willhite and Blanke, 2004), which may be associated with changes in the permeability of the mitochondrial membrane, allowing release of cytochrome c from the mitochondrion. Experiments performed with VacA-P9A and VacA-G14A (well characterized mutant forms of VacA which are unable to form membrane channels), clearly showed that VacA membrane channel formation is essential for reduction of the mitochondrial membrane potential and subsequent cytochrome c release.

The mechanism by which VacA permeabilizes the mitochondrial membrane is still unknown. One model suggests that VacA directly permeabilizes the mitochondrial inner membrane by inserting and forming pores, which could result in a reduction in mitochondrial transmembrane potential leading to further changes in the outer membrane and release of cytochrome c into the

cytosol. Alternatively, it has been suggested that VacA could activate endogenous channels of the mitochondria such as the voltage dependent anion channel (VDAC), an abundant protein located in the outer membrane of mitochondria, which is thought to facilitate cytochrome c release (Tsujimoto *et al*., 2002).

Interestingly it has been observed that the ability of VacA to induce apoptosis seems to be cell-type dependent, since no apoptosis could be detected in VacA-treated activated T cells (Gebert *et al*., 2003, Boncristiano *et al*., 2003) but VacA induces apoptosis in parietal cells (Neu *et al*., 2002). This latter effect might facilitate *H. pylori* colonization of the gastric mucosa.

1.3.8.5 Effects of VacA on cells of the immune system

The vacuolating cytotoxin displays a diverse repertoire of novel activities for combating the immune defence mechanisms of the host. VacA has been shown to inhibit antigen presentation in B cells. This is thought to occur by blocking the maturation of endosomes to MHC class II compartments, where antigen loading takes place (Molinari *et al*., 1998b). Vaccination studies in animal models, (Ermak *et al*., 1998) and adoptive transfer experiments of T cells, (Lucas *et al*., 2001) have suggested that CD4+ T cells are a putative target of manipulation by *H. pylori* during infection. VacA, when added to T cells has been shown to completely inhibit the ability of human peripheral blood lymphocytic cells (PBLC) to proliferate (Gebert *et al*., 2003). In addition to inhibition of proliferation, VacA has also been found to inhibit secretion of

interleukin-2 (IL-2), which is essential for T cell proliferation and viability (Gebert *et al*., 2003). Furthermore VacA has been observed to down regulate expression of IL-2Rα, a high affinity receptor expressed by T cells (Boncristiano *et al*., 2003). Most of these effects of VacA on T cells are expected to result in localized immune suppression. It has been suggested that VacA enters the lamina propria via disruptions in the epithelial cell layer leading to intoxication of immune cells.

In epithelial cells, *H. pylori* seems to enter large cytoplasmic vacuoles, where the bacteria remain viable and motile. In professional phagocytes, such as macrophage cell lines THP-1 (human) and RAW 264.7 (murine), *H. pylori* displays an enhanced survival, as compared to a *vacA*-negative mutant strain (Zheng and Jones, 2003). VacA producing strains appear to reside in a compartment that has properties of an early endosome and avoids fusion with lysosomes. VacA is thought to arrest phagosome maturation by recruiting and retaining tryptophane aspartate-containing coat protein (TACO). This is a mechanism also used by Mycobacterium bovis to prevent phagosome trafficking and maturation (Ferrari *et al*., 1999). However, the role of VacA in phagosome fusion is uncertain (Rittig *et al*., 2003). VacA has also been reported to stimulate apoptosis in macrophages (Menaker *et al*., 2004). VacA has been shown to upregulate the expression of chemokines in eosinophils (Kim *et al*., 2007). VacA has also been shown to increase COX-2 expression and activates p38 MAP kinase in neutrophils (Boncristiano *et al*., 2003, Brest *et al*., 2006). Incubation of VacA with mast cells has been shown to stimulate

proinflammatory cytokine production and induce mast cell chemotaxis (Supajatura *et al*., 2002, de Bernard *et al*., 2005).

1.4 Regulation of gene expression

In general, protein production is regulated in a number of ways; these are discussed in detail generally and with respect to *H. pylori* below.

1.4.1 Regulation of transcription Initiation

Bacteria are highly versatile and responsive organisms that are able to respond to environmental changes such as heat shock, cold shock, acid shock, starvation and nutrient depletion by altering gene expression. This regulation aids the survival of the organism under these altered and stressful conditions.

Gene expression is the combined process of the transcription of a gene into mRNA, the processing of that mRNA, and its translation into protein. It is generally observed that most regulation of bacterial protein expression occurs at the transcriptional level as it is most energetically economical for an organism to terminate expression of that gene and therefore block production at the earliest possible stage if demand for a protein decreases. However, other stages of transcription are also targets for regulation in some cases.

One of the most striking features of the *H. pylori* genome is the relatively low abundance of predicted regulatory proteins. Only 32 gene products classified as having a possible regulatory function have been identified. Further analysis of

the genome has revealed that only 17 loci are predicted to have a role in the regulation of transcription in *H. pylori* (Scarlato *et al*., 2001).

1.4.1.1 Sigma factors

Sigma (σ) factors are key transcriptional activators which form part of the RNA polymerase holoenzyme. Generally, a sigma subunit is required for the bacterial RNA polymerase to recognise a given promoter. Many bacteria make several different sigma subunits each of which can interact with the RNA polymerase core to form the holoenzyme and direct it to a specific set of promoters. This scheme permits large sets of genes to be differentially regulated by simply replacing one sigma subunit with another. The principle sigma factor in *E. coli* is σ70 so called because the protein is 70 kDa in size. It is used for normal metabolic "housekeeping" functions.

When exposed to stressful conditions, alternative sigma factors are used to allow the expression of genes which allow the bacterium to survive under these new stressful conditions. σ32 is required for the production of heat shock proteins (HSPs) which act as chaperones to protect cells against heat damage and other stress situations as well as preventing mis-folding of proteins (Hesterkamp and Bukau, 1998). Some parts of the sequence of σ32 can be recognized in other bacterial sigma factors. σ54 is present all the time and it is required for the expression of many genes that are involved for nitrogen metabolism. σ54 has a very different sequence and structure than the other sigma factors in *E. coli* and most bacteria; thus there are at least two different

families of sigma factor proteins in bacteria. σ38 (or σs) is known to be associated with a variety of stress responses upon entry into stationary phase including osmotic stress (Hengge-Aronis, 2002b, Hengge-Aronis, 2002a). The importance of σ38 has been increasingly recognised in recent years. This sigma factor appears only as cells enter the stationary phase of growth. It is responsible for transcription of all of the genes whose products are required during stationary phase.

H. pylori possesses only three sigma factors: σ80, σ54 and σ28. σ80 is the "normal" housekeeping sigma factor in *H. pylori*. Because of the resemblance to the *E. coli* protein, this factor was designated σ70 by some authors (Tomb *et al*., 1997, Solnick *et al*., 1997), while based on the predicted molecular mass – σ80 by others (Beier *et al*., 1998). Recombinant *H. pylori* σ80 expressed in *E. coli* is fully functional but *H. pylori* promoters have higher specificity for *E. coli* RNA polymerases utilising *H. pylori* σ80 above *E. coli* σ70 (Beier *et al*., 1998, Spohn and Scarlato, 1999a). The specificity of σ80 to *H. pylori* promoters has been attributed to the spacer region between two domains in the *H. pylori* σ80. *H. pylori* σ80 mutants could not be isolated, and so it is assumed that an intact σ80 is necessary for the viability of *H. pylori*. σ54 and σ28, the alternative sigma factors both seem to be dedicated exclusively to the transcriptional regulation of flagellar genes (Spohn and Scarlato, 1999a, Suerbaum *et al*., 1993, Kim *et al*., 1999).

Mapping of the transcriptional start sites and identification of promoter sequences of *H. pylori* genes has implicated the role of these factors in

transcriptional regulation of different sets of genes (Akada *et al*., 2000, Beier *et al*., 1997, Bereswill *et al*., 1998, Burns *et al*., 2000, Dundon *et al*., 1999, Heuermann and Haas, 1995, Leying *et al*., 1992, Lundstrom and Bolin, 2000, Odenbreit *et al*., 1999, Pesci *et al*., 1994, Porwollik *et al*., 1999, Schmitz *et al*., 1997, Spohn and Scarlato, 1999b, Spohn and Scarlato, 1999a, Suerbaum *et al*., 1993). *H. pylori* does not possess homologues of either the stationary phase sigma factor (σ38) or the heat-shock sigma factor (σ32). The absence of these factors suggests that *H. pylori* has a different mechanism to stress response than other bacteria.

Analysis of consensus promoter sequences and RpoD in *H. pylori* revealed that there were no obvious -35 consensus sequence in *H. pylori* genes *vacA, katA, cagB p2, cheY, ureA, cagA, hspA, cagB P3, copA, repA, sodB.* The absence of a consensus *H. pylori* -35 sequence led to the speculation that there may be structural differences between *H. pylori* RpoD (Solnick *et al*., 1997). The DNA binding domains of RpoD from *H. pylori* 26695 (Tomb *et al*., 1997) were aligned with the corresponding domains of RpoD from 10 other bacterial genera. Alignment of the domains responsible for binding to the -10 motif indicates *H. pylori* differs from the RpoD consensus sequence at 4 positions. A more striking degree of divergence occurred in the 4.2 domain of the *H. pylori* RpoD, which typically mediates binding to -35 promoter elements. This is consistent with the finding that -35 promoter elements in *H. pylori* that are quite different to those found in *E. coli*.

Lack of conservation between *H. pylori* promoters outside the -10 hexamer suggest that there may be considerable variation in the avidity of RpoD binding to different promoters and may represent a mechanism for determining the levels at which individual genes are constitutively expressed.

1.4.1.2 Transcription factors

Analysis of the *H. pylori* genomes of strains 26695 (Tomb *et al*., 1997) and J99 (Alm *et al*., 1999) indicates that only two proteins with homology to known and characterised repressors have been identified. These include the homologues of the ferric uptake regulatory protein (Fur), and the heat shock regulator (HspR). Further analysis led to the identification of the carbon storage regulator (CsrA) and the nickel uptake regulator (NikR). There are three additional possible transcriptional repressors: one is a homologue of the BirA repressor of the *E. coli* biotin operon; another is a homologue of the *Bacillus subtilis* chromosome partitioning protein Soj, which has been shown to exert a direct responsive action on promoters of different sporulation genes (Quisel *et al*., 1999). The most recently described is a homologue of the HrcA repressor of *B. subtilis*, which controls chaperone gene expression (Spohn and Scarlato, 1999a, Homuth *et al*., 2000).

The ferric uptake regulator (Fur) of *E. coli* is a 17kDa polypeptide which acts as a transcriptional repressor of iron-uptake and metabolism genes by means of its iron-dependent DNA-binding activity. When high concentrations of iron are present, Fur binds ferrous iron and undergoes a conformational change which

enables it to bind its target sequences on the DNA (Fur boxes), and repress transcription. Low iron concentrations lead to release of ferrous iron from the protein, and the resulting decreased DNA-binding affinity leads to derepression of transcription, and hence protein expression.

Iron acts as a corepressor of *H. pylori* Fur proteins. Several Fur boxes have been found in the promoter regions of different iron-uptake and iron–detoxification genes (Tomb *et al*., 1997, Odenbreit *et al*., 1996). Iron-mediated repression has been shown for *fecA2* (Bereswill *et al*., 2000) and *ribBA* (Worst *et al*., 1998) and has been investigated for the *vacA* and fur genes (Szczebara *et al*., 1999). Fur has also been shown to be necessary for iron-dependent regulation of transcription of the *pfr* gene which codes for a ferritin-like protein involved in intracellular iron storage (Bereswill *et al*., 1998, Bereswill *et al*., 2000). Expression of *pfr* is repressed under low iron conditions in wild-type *H. pylori* but not in an isogenic *fur*- mutant, which indicated that Fur acts as a transcriptional repressor of this gene in the response to iron deprivation (Bereswill *et al*., 2000).

In addition to regulation of iron acquisition, Fur also plays a central role in the acid tolerance response (Audia *et al*., 2001). *H. pylori* Fur protein was found to be directly or indirectly involved in the acid induction of urease, amidase and formidase (Bury-Mone *et al*., 2004; Heimer *et al*., 2002). Furthermore, the presence of active Fur protein in *H. pylori* is essential for resistance to acidity *in vitro* (Bijlsma *et al*., 2002). Moreover, Fur is required for efficient colonization of the Mongolian gerbil (Gancz *et al*., 2006).

Using assays of DNA binding activity, it has been shown that Fur can specifically bind to a number of promoters. However, promoters bound by Fur appear to be regulated differently. While the *fur* promoter is activated by the Fur protein, other promoters are repressed under low-iron conditions and others appear to be iron-independent (Delany *et al*., 2001a, Delany *et al*., 2002b, Delany *et al*., 2001b, Delany *et al*., 2002a, Spohn *et al*., 2002). Therefore Fur protein acts as a transcriptional activator on its own promoter, and as a repressor on other promoters.

The global transcriptional regulatory repressors like the HspR repressor in *Streptomyces* species allows the bacterium to respond to heat shock (Bucca *et al*., 1995, Bucca *et al*., 1997). HspR binds to HspR-associated inverted repeat (HAIR) motifs found in the upstream promoter regions of chaperone genes. At physiological temperature this binding appears to be stable, leading to repression of transcription. An increase in temperature destabilises the protein and/or the protein-DNA interaction, which results in derepression of transcription of the chaperone genes (Grandvalet *et al*., 1999).

The *H. pylori* genome contains homologues to common heat shock proteins such as *groEL*, *groES*, *dnaK* and *cbpA* (Tomb *et al*., 1997, Spohn and Scarlato, 1999b). GroEL, GroES and DnaK have been implicated in regulating urease activity and adhesion to epithelial cells (Suerbaum *et al*., 1994; Yamaguchi *et al*, 1997). *H. pylori* does not possess a σ32 homologue, but homologues of repressors such as HspR and HrcA have been identified (Tomb *et al*., 1997,

Spohn and Scarlato, 1999a). The *hspR* gene of *H. pylori* is the second gene in an operon which contains *cbpA* (encoding a DnaJ homologous protein) and a gene coding for a putative helicase-like protein. HspR has been shown to repress transcription of this operon as well as *groES* and *dnaK* (Spohn and Scarlato, 1999a), by binding to large DNA regions of approximately 75bp in the respective promoter regions. All three binding sites were found to contain sequences with similarities to the HAIR consensus sequence established for *Streptomyces*, indicating similar DNA binding specificities of both proteins. Transcriptional analysis of the major heat-shock genes of *H. pylori* using Northern blot analysis has detected increased amounts of both *groESL* and *dnaK*- associated RNAs after 30 minutes of temperature up shift to 42˚C (Homuth *et al*., 2000). Transcription of the *cbp-hspR-orf* and *groESL* operons has been demonstrated in response to osmotic shock (Spohn and Scarlato 1999a). In addition Barnard *et al* (Barnard *et al*., 2004) demonstrated that transcription of *groESL* and *hspR* were up regulated in response to a temperature up shift. A HrcA homologue has also been identified in *H. pylori* and is encoded by the first gene of the *dnaK* operon. It has been speculated that the HrcA homologue could function as a corepressor of the *groESL* and *dnaK* promoters together with the HspR protein. In support of this hypothesis it has been found that the *groESL* and *dnaK* promoters contain sequences with similarities to the controlling inverted repeat of chaperone expression consensus sequence, which has been established as the binding site for the HrcA protein of *Bacillus subtilis*. As *H. pylori* does not possess a σ32 homologue it has been speculated that HspR and HrcA may act in co-

ordination with each other to regulate gene expression of *H. pylori* chaperone genes.

NikR is a nickel responsive transcription factor, which has been found to control the activation and repression of several genes including urease, the outer membrane proteins FecA3 and FrpB4 (van Vliet *et al*., 2004a, van Vliet *et al*., 2002, van Vliet *et al*., 2004b, Bury-Mone *et al*., 2004, Ernst *et al*., 2005b, Wolfram *et al*., 2006, Contreras *et al*., 2003). NikR has also been shown to be involved in the control of expression of several genes in response to acidic conditions in *H. pylori* (Bury-Mone *et al*., 2004, van Vliet *et al*., 2004a, van Vliet *et al*., 2004b). The NikR acid-responsive regulatory circuit has been found to functionally overlap with the Fur regulator (Bury-Mone *et al*., 2004, van Vliet *et al*., 2004a, van Vliet *et al*., 2004b).

The Carbon storage regulator A (CsrA) is a post-transcriptional regulator (Romeo, 1998, Romeo and Gong, 1993) that appears to be involved in influencing the processing and translation of target transcripts in *H. pylori* but has minimal effects on the stability of transcripts (Barnard *et al*., 2004). CsrA has been shown to be involved in modulating motility and plays a role in controlling gene expression in response to environmental stress (Barnard *et al*., 2004) and has been shown to affect Fur and HspR expression. Mutagenesis of *csrA* in *H. pylori* resulted in the deregulation of the acid induction of *vacA* (Barnard *et al*., 2004). Furthermore, CsrA appears to play a role in *H. pylori* colonisation as deletion of CsrA inhibited the strains ability to colonise a mouse model of infection (Barnard *et al*., 2004).

Transcription profiling studies have revealed that there is considerable overlap between regulatory responses to growth phase-regulated genes, regulatory responses to acid, nickel and iron restriction in *H. pylori* (Bury-Mone *et al*., 2004, Ernst *et al*., 2005a, Merrell *et al*., 2003a, Merrell *et al*., 2003b, van Vliet *et al*., 2004a, van Vliet *et al*., 2004b, Wen *et al*., 2003).

1.4.2 Two-component systems

Two-component systems are a method by which bacteria regulate their cellular functions in response to changing environmental conditions. They are composed of a sensor protein, also referred to as histidine kinase which detects environmental stimuli by its N-terminal input domain, and a cognate response regulator, which elicits a cellular response via its C-terminal output domain, frequently by acting as a transcriptional activator. The response regulator is activated by a phosphotransfer reaction from the histidine kinase, which autophosphorylates in the presence of the appropriate environmental stimulus, to an aspartic acid residue in the N-terminal receiver domain of the regulator protein. Phosphorylation of the receiver domain then triggers a conformational change in the response regulator which activates its C-terminal output domain. *H. pylori* has a relatively small array of these two-component systems (Tomb *et al*., 1997). Besides the CheA/CheY (HP0392/HP1067) system that regulates the chemotactic response, there are only three histidine kinases and five response regulators putatively involved in transcriptional regulation (Joseph and Beier, 2007). There are relatively few two-component systems found in *H.*

pylori (*E. coli* encodes approximately 30 two-component systems (Inui *et al*., 2003) and *Campylobacter jejuni*, a close relative of *H. pylori* encodes seven two-component systems, as well as three orphan response regulators (Parkhill *et al*., 2000).) This small number of regulatory proteins may be due to a lesser need for *H. pylori* to be able to adapt to environmental changes because of the restricted niche it inhabits and the lack of competition from other organisms.

Motility and urease dependent acid resistance required for colonisation of the stomach are regulated by 2 component systems. In *H. pylori*. HP0703-HP0244 (FlgRS) is a two component system protein and is part of the regulatory cascade involved in the regulation of class II flagellar gene expression (Niehus *et al* 2004). HP0392-HP1067 (CheAY2-CheY) is a two component system protein and a bifunctional histidine kinase and is involved in chemotaxis and regulation of flagellar motion. HP0019 (CheV1), HP0616 (CheV2), HP0393 (CheV3) are response regulators that are thought to be involved in chemotaxis but their precise function is unknown. HP0166-HP0165 (ArsRS) is a two component system protein and an essential response regulator and is involved in the pH-responsive regulation of the urease gene cluster and other genes involved in acid resistance (Pflock *et al*., 2005, Pflock *et al*., 2006, Wen *et al*., 2006). HP1365-HP1364 (CrdRS) is a two component system protein and is possibly involved in the regulation of genes encoding Cu^{2+} resistance determinant regulation of acid resistance genes. Waidner *et al*., 2005 demonstrated that CrdRS positively regulates the expression of the copper resistance determinant CrdAB-CzcAB in response to increasing concentrations of copper ions (Waidner *et al*., 2005).

1.4.3 Transcriptional termination

Once RNA polymerase has initiated transcription at a promoter, it will continue along the DNA, polymerizing ribonucleotides, until it encounters a transcription termination site in the DNA at the end of the cluster of genes being transcribed. Bacterial DNA has two basic types of transcription termination sites: factor-independent and factor-dependent. As their names suggest, these types are distinguished by whether they work with just RNA polymerase and DNA alone or need other factors before they can terminate transcription.

1.4.3.1 Factor-independent termination

Factor-independent terminator sequences are characterized by the presence of self-complementary sequences at the 3' end of genes causing hairpin structures in the RNA which act as terminators. The stem of the hairpin often has a high content of G-C base pairs giving it high stability, causing the polymerase to pause. The hairpin is often followed by four or more uracil residues which result in weak RNA-antisense DNA strand binding. This favours dissociation of the RNA strand, causing transcription termination.

1.4.3.2 Factor-dependent termination

While RNA polymerase can self-terminate at a hairpin structure followed by a stretch of U residues, other known terminator sites may not form strong hairpins, so require an accessory factor for efficient transcription termination.

The transcription termination factor Rho (ρ) is thought to exist in all types of bacteria, and is the most widely understood of the transcription factors. Rho is a hexameric protein that binds to specific sites in single stranded RNA. It hydrolyzes ATP and moves along the nascent RNA towards the transcription complex, where it enables the polymerase to terminate transcription at Rho-dependent transcriptional terminators. Like the factor independent terminators, these signals are recognized in the newly synthesized RNA rather than in the template DNA.

Genome sequence analysis comparisons have revealed that *H. pylori* has very few stem loop structures; therefore it is presumed that transcription termination occurs primarily via a Rho- dependent mechanism (Washio *et al*., 1998). This is supported by the discovery of *H. pylori* orthologues to known *E. coli* transcription termination factors (rho, nusA, nusB and nusG) (Bandyopadhyay *et al*., 2001).

1.4.3.3 Attenuation and antitermination

Bacteria use mechanisms known as attenuation and antitermination to regulate transcription elongation in order to control gene expression in response to changes in their environment. Both involve controlling the formation of a transcription terminator structure in the RNA transcript prior to a structural gene or operon. Transcription attenuation is a mechanism that utilizes transcription pausing to regulate the expression of downstream genes in response to environmental changes. Antitermination is generally distinguished

from attenuation in that the action of the regulatory molecule results in transcription read through, with the default pathway being premature termination; whereas in attenuation, the regulatory molecule induces transcription termination and the default pathway is read through. Several different mechanisms of attenuation and antitermination have been identified, but the best known mechanism is that used in *E. coli* for attenuation at the *trp* operon. The 5' end of the *trp* operon mRNA is rich in tryptophan codons. At high tryptophan levels, normal translation of this sequence occurs. As this happens, the *trp* mRNA forms a terminator hairpin structure that does not allow RNA polymerase to continue the transcription of the rest of the *trp* operon. Therefore under normal conditions, most *trp* operon transcripts are attenuated. When the tryptophan level drops the ribosome stalls when trying to translate the 5' end of the *trp* operon, and results in the formation of an antiterminator hairpin structure in the next segment of *trp* mRNA, which allows the RNA polymerase to continue with the transcription of the *trp* operon (Kuroda and Yanofsky, 1984). Other mechanisms of antitermination rely on RNA binding proteins that bind and stabilise an antiterminator hairpin structure which prevents early termination of transcription e.g. the *blg* operon in *E.coli* (Amster-Choder *et al*, 2005). Other mechanisms of antitermination have also been described that rely on regulatory proteins that bind to RNA and interact with RNA polymerase to prevent the RNA polymerase from recognising RNA terminators e.g N protein-mediated antitermination in bacteriophage λ (DeVito *et al*., 1994). No mechanisms of attenuation or antitermination have yet been described in *H. pylori*.

1.4.3.4 mRNA stability

The wide variation in mRNA stability in both prokaryotes and eukaryotes suggests that the decay rate of mRNA is involved in the control of gene expression. The complex mechanisms of degredation that determine the stability of mRNA in bacteria have been investigated extensively in the last decade and are beginning to be understood. The vast majority of mRNAs in a bacterial cell are very unstable, having a half-life of about 3 minutes. Endoribonucleases are usually responsible for the initial cleavage of the transcript allowing exonucleases to degrade the cleavage products in the 3' to 5' direction. Because its mRNAs are both rapidly synthesized and rapidly degraded, a bacterial cell can adapt quickly to environmental changes.

H. pylori encodes at least three genes involved in RNA processing (*papS* (polyadenylate polymerase), *rnc* (RNase III), *rnpA* (RNase P)) and one involved in RNA degradation ((*pnp*) polynucleotide phosphorylase) (Tomb *et al*., 1997). The products of these genes are likely to be part of the degradosome of *H. pylori*. Therefore, turnover of RNA is expected to be a mechanism regulating gene expression in *H. pylori*. The first evidence that mRNA stability regulates gene expression in response to changes in environmental conditions came from studies of the urease operon of *H. pylori* (Akada *et al*., 2000). It was demonstrated that the urease gene cluster of *H. pylori* consists of two operons, *ureAB* and *ureIEFGH*, and that primary transcripts of *ureIEFGH* as well as of the read-through transcript, *ureABIEFGH*, are cleaved to produce several species of mRNA. The finding that mRNA from this operon was found to be

less stable at pH 7 than at pH 5.5, suggests that the *ureIEFGH* operon is regulated post-transcriptionally by mRNA decay in response to environmental pH (Akada *et al*., 2000).

In *E. coli*, the 5' untranslated region of the *ompA* transcript contains a stem-loop structure at the extreme end of the 5' terminus that has been shown to stabilise mRNA transcripts, and protects mRNA from degradation (Emory *et al*., 1992). The potential to form a hairpin structure close to the 5' end is a feature common to a number of stable prokaryotic messages. Sequence alignments of a collection of *H. pylori* strains has revealed a potential stem-loop structure at the +4 site of the *vacA* 5'-untranslated region. It is not clear if this potential stem loop structure has an effect on transcription or transcript stability; although deletion caused a reduction in transcript level (Narayanan, 2005). The role of mRNA stability in the regulation of VacA production will be discussed in detail in chapter 5.

1.4.4 Translational regulation

As previously mentioned most regulation of protein production takes place at the transcriptional level, a point at which it is most economic to terminate protein production if a given protein is no longer required. However, there are additional mechanisms of regulation that occur at the translational level.

Efficient initiation of translation depends on the existence of a ribosome binding site (a purine rich group of six to eight nucleotides also known as the

Shine-Dalgarno sequence) just upstream of the AUG start codon. The ribosome binding site has close complementarity in sequence to the 3' end of the *16S rRNA* molecules found in bacterial ribosomes. Initial positioning of mRNA upon the smaller ribosomal subunit requires base pairing between the *16S rRNA* chain and the ribosome-binding (Shine-Dalgarno) sequences. Mutations in the ribosome binding sequence including mutations that cause this sequence to be nearer or further away from the AUG codon can greatly reduce translational efficiency, compared to those mRNAs that have their ribosome-binding sequences centred eight nucleotides upstream from the AUG start codon, which are translated efficiently.

The efficiency with which Shine-Dalgarno sequences work can be mediated by proteins that bind to them and block their availability. The best understood example of this involves the ribosomal (r-proteins) of *E.coli*. When the rate of r-protein synthesis exceeds the rate of rRNA synthesis, free r-proteins accumulate. Some of them bind to the Shine-Dalgarno sequences on the r-protein mRNAs and prevent further translation, so that the rate synthesis of ribosomal proteins does not exceed the rate at which they can be used in making ribosomes. When r-protein mRNAs are rendered untranslatable by the binding of r-proteins, they are degraded more rapidly than usual. Once translation is underway, its rate is determined by the availability of the various tRNA species that correspond to the specific codons in use in the mRNA molecules. Different tRNA molecules are present in different amounts; more commonly used codons generally have larger quantities of the corresponding tRNA molecules. mRNA with a high proportion of codons recognised by rare

tRNA molecules are therefore translated more slowly than codons recognised by abundant tRNAs. In addition to regulation by r-proteins, regulation of translation initiation can also be regulated by the formation of an RNA hairpin that sequesters the ribosomal binding site (RBS) of the mRNA and prevents translation initiation. A good example of this mechanism is the expression of the cobalamin (*cbl)* biosynthetic cob operon in *Salmonella typhimurium* (Ravnum and Andersson, 2001). In the absence of cobalamin a cis-acting translational enhancer element, located 83 nucleotides upstream of the Shine-Dalgarno sequence in the 5'-untranslated region of the *cob* mRNA, is required to unfold the inhibitory RBS hairpin. In the absence of cobalamin the translational enhancer element, which consists of 5 nucleotides, functions as an enhancer by interacting with nucleotides in the stem of the RBS hairpin. This interaction destabilizes the RNA hairpin and allows ribosome binding. In the presence of cobalamin, the enhancer function is inhibited. As a result, the RBS hairpin forms and prevents translation initiation (Ravnum and Andersson, 2001).

From genome analysis of the *H. pylori* strain 26695, the translational machinery appears similar to that of the well characterised *E. coli* system. There are three genes associated with initiation factors of translation, four genes involved with elongation and three genes coding for release factors.

1.4.5 Phase-variation

In contrast to transcriptional regulation, Phase-variation allows the continuous generation of alternative phenotypes within a population, which facilitates adaptation to changing environmental conditions and allows the organism to evade detection by the host immune system by creating antigenic variation of surface exposed epitopes of flagella, adhesins and lipopolysaccharide (LPS). Phase variation allows reversible switching between phenotypes, by a genetic re-organisation, mutation or modification, which is not associated with a loss of coding potential. A well known mechanism is slipped strand mispairing of DNA, which occurs in short sequence repeat (SSR) regions of DNA present in the target genes (van Belkum, 1999). These repetitive sequences can consist of single or multi-nucleotide repeat sequences and are positioned upstream of a gene which affects transcription or within a gene which affects translation (Henderson *et al*, 1999). The number of repeats keeps the gene in frame and if the number of repeats increases or decreases during cell replication, the translation of the gene will be out of frame leading to a phenotypic change. The repetitive sequences usually consist of poly-purines or pyrimidines which form a triple stranded form of DNA along with a single stranded form of DNA which may be more likely to form under conditions of stress. This form of DNA may stimulate gene expression if located near a 5′ end of a promoter region and the single stranded region may promote slipped strand mispairing (Henderson *et al*, 1999).

The LPS of most *H. pylori* strains contain complex carbohydrates known as Lewis antigens that are structurally related to the human blood group antigens.

H. pylori fucosyltransferases (FUCTs) are responsible for expression of alternative Lewis antigens in a given strain. The presence of some cis-elements in the FUCT encoding genes, including the polyA-polyC tract, mediate slipped strand mispairing and the signature sequences regulating the -1 translation frameshifting allow *H. pylori* to produce interchangeable Lewis antigens. Genome sequencing has revealed that *H. pylori* possesses many sequence repeat regions (Alm *et al*., 1999, Tomb *et al*., 1997). Forty six putative phase variable genes have been identified which have the potential to act by altering the translational frame of the coding regions. These putative phase variable genes include genes involved in LPS biosynthesis, production of cell surface proteins, and DNA restriction modification systems (Salaun *et al*., 2004, de Vries *et al*., 2001) (Henderson and Owen, 1999). As the *H. pylori* genome is relatively small, the abundance of repetitive DNA suggests that phase-variation may offer an efficient way of altering gene expression (de Vries *et al*., 2001).

1.4.6 Protein secretion

Many secreted bacterial proteins possess a signal sequence in their N-terminus, which directs secretion by the general secretion pathway. The signal peptidase then cleaves the signal sequence from the N-terminus of membrane and secreted proteins after they have been translocated across the cytoplasmic membrane. Bacterial signal sequences possess a positively charged N-terminus that orientates the signal in the inner membrane, a hydrophobic core that forms a membrane spanning helix, and a cleavage recognition site that acts as a substrate for the leader peptidase. Analysis of a large number of signal

sequences has shown there is a characteristic pattern of small apolar residues at positions -1 and -3 (with respect to the cleavage site) and a helix breaking residue in the region -4 to -6. It has been observed that these positions are the most important with respect to processing, with positions -2, -4, -5 and +1 being of secondary importance (von Heijne, 1983, Perlman and Halvorson, 1983). Such structural regularity in the -3 and -1 region is considered to be necessary for recognition of the signal peptide cleavage site by leader peptidase. As mentioned previously VacA type s1 and VacA type s2 not only differ in the position of their signal cleavage site (with type s2 possessing a 12-amino acid hydrophilic N-terminal extension (Letley *et al*., 2003, Letley and Atherton, 2000), but also in the amino acids present at various positions within the signal sequence (Figure 1.5.). Both VacA type s1 and s2 have alanine present at the -1 position. Studies on the processing of *E. coli* alkaline phosphatase, show that the presence of alanine, glycine and serine at -1 position allows processing, with alanine providing the highest rate of processing (Karamyshev *et al*., 1998). Serine is present at position -3 in type s1 VacA, whereas type s2 VacA has leucine present at this position. Studies on the effect of sequence variation on the processing of the M13 procoat protein revealed that, only procoat proteins with small amino acids (serine and glycine) at the -3 position were efficiently cleaved *in vivo*, whereas presence of leucine at the -3 position retarded cleavage (Shen *et al*., 1991). However it has been suggested that the effect of some amino acid substitutions at position -3 on processing may vary from protein to protein, as mutant maltose-binding proteins with leucine, threonine or valine at the -3 position were processed efficiently (Fikes *et al*., 1990).

A characteristic feature of bacterial signal sequences is the presence of a helix-destabilizing residue such as a proline or glycine between the -4 and the -6 position of the peptide. In type s1 VacA, proline is present at -6, whereas in type s2 glycine is present at this position. Although proline and glycine are both effective helix breakers, procoat substituted with a glycine at the -6 position was not processed *in vivo*, and had reduced processing *in vitro*, compared to the wild type with proline at -6 (Shen *et al*., 1991). It is thought that proline helps alter the conformation of the polypeptide chain surrounding the cleavage site, allowing efficient cleavage of the signal peptide. In addition to the differences between VacA type s1 and s2 at the -1, -3, and -6 positions, it has also been observed that there are differences in the signal sequences between VacA type s1a strains that possess a glutamine at the -4 position and, type s1b VacA that has glutamate at -4. However a study of the processing of mutant alkaline phosphatases showed that only lysine when present at the -4 position affects processing (Karamyshev *et al*., 1998). The effect of differences in signal peptide processing on VacA production will be discussed in detail in chapter 6.

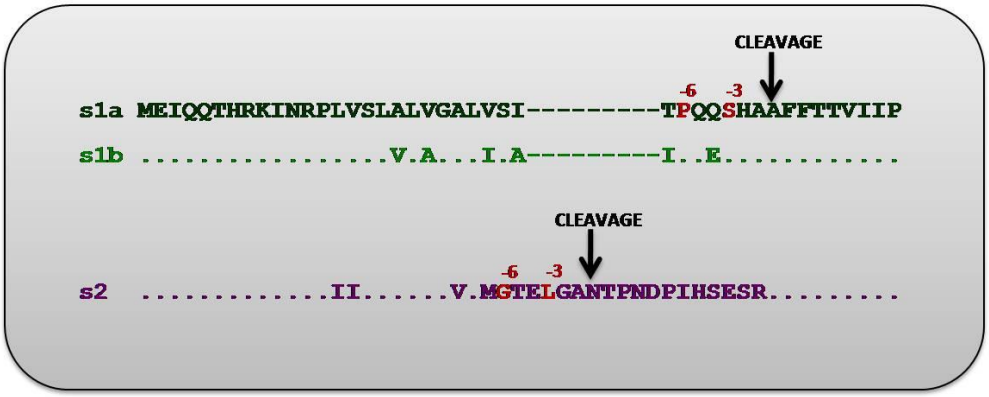


Figure 1.5 Signal sequences of VacA type s1a, s1b and s2. The cleavage site of each sequence is indicated by an arrow. Type s2 VacA possesses a 12-amino acid N-terminal extension and so differs in its cleavage site to s1 type *vacA*. All three signal sequences possess an alanine at the -1 position. Type s1a and s1b, VacA have a serine at position -3 and a proline at the -6 position, whereas type s2 VacA has leucine at position -3 and a glycine at position -6.

1.5 Determinants of VacA production

1.5.1 Genetic determinants of *vacA* expression

Heterogeneity in VacA levels are known to exist between strains. Forsyth *et al.,* (1998) first described key findings focussing on the heterogeneity of *vacA* levels amongst different *H. pylori* strains. It was observed that yields of VacA were higher among s1/m1 strains than s2/m2 strains. Using primer extension analysis it was concluded that differences in VacA levels between strains could be attributed to differences in *vacA* transcription (Forsyth *et al*., 1998).

Previous studies have shown that *vacA* transcription is initiated by a single promoter which is located in the 285bp intergenic region between the upstream housekeeping gene, cysteinyl-tRNA synthase (*cysS*) and the start of *vacA* (Forsyth *et al.,* 1998). Sequence analysis of this region from 12 strains (Forsyth *et al.,* 1998) revealed that *vacA* has a single TSP which is located 119 nucleotides upstream of the AUG start codon, a conserved -35 region (TTTATG), a conserved -10 region (TAAAAA) and a TGN motif adjacent to the -10 motif (Davies *et al.,* 2002) similar to the extended -10 boxes found in *E. coli* (Forsyth *et al.,* 1998). Site directed mutagenesis indicated that -35 and extended -10 sequences along with promoter element spacing are important in determining *vacA* transcript levels. Differences in *vacA* expression between 8 strains *in vitro* were shown to be unrelated to -10 and -35 sequences (Ayala *et al.,* 2004). G. L. Narayanan (2005) analysed the *vacA* promoter sequences of a collection of published strains and found, in agreement with Forsyth *et al* (1999) that there appears to be an extended – 10 region. She also found that in

4 out of 8 clinical strains there was a substitution from a G to an A at position -14 that makes up part of the extended -10 region. In addition the final base within the -35 motif has a frequent substitution from a G to an A residue. An inverted repeat upstream of the -35 motif was found to be present in 4 out of 8 clinical strains (Ayala *et al.,* 2004). Inverted repeats are a common feature of regulator binding sites, however the role of the inverted repeat in the *cysS – vacA* intergenic region in transcriptional regulation of *vacA* is yet to be determined.

In order to determine whether different promoter strengths between strains was the reason for differing transcriptional levels between s1/m1 and s2/m2 strains, comparison of promoter regions and promoter exchange experiments were performed between an s1/m1 strain (60190) and an s2/m2 strain (86-313). These suggested that there is a difference in promoter strength between s1/m1 strains and s2/m2 strains, but that factors which govern this difference in promoter strength must be located outside the promoter region. As *vacA* from an s2/m2 strain was capable of yielding higher levels of transcription in an s1/m1 background, this suggested that either s1/m1 strains produce an activator of transcription, or s2/m2 strains produce a repressor of transcription (Forsyth *et al*., 1998, Forsyth and Cover, 1999).

An alternative suggestion put forward by Forsyth *et al.* is that *vacA* mRNA transcript stability differs between the two strains, but experimental data was not obtained. Comparison of the 3' ends of *vacA* transcripts revealed that both s1/m1 and s2/m2 strains possessed prominent stem-loop structures at the 3'end,

thus indicating that there were no differences in transcript structures that could account for differences in *vacA* levels. Furthermore, in the promoter exchange experiments, the entire 5' untranslated regions were swapped from the s1/m1 strain to the s2/m2 strain, yet there was no significant increase in *vacA* mRNA levels (Forsyth *et al*., 1998). A sequence alignment of the published promoter regions of 19 *H. pylori* strains revealed a conserved stem loop structure at the +4 position of the mRNA transcript. Deletion of this stem loop resulted in a reduction in *vacA* mRNA levels compared to the control. The alignment also revealed that this stem loop was reasonably well conserved except for differences at the +11 and +28 positions within the stem loop. However, the importance of these polymorphisms in determining *vacA* transcript level was not determined G. L Narayanan (2005).

1.5.2 Environmental determinants of *vacA* expression

Several studies have shown that VacA levels are also influenced by environmental stimuli. Microarray analysis has demonstrated that *vacA* expression is repressed after acid exposure at pH 5.0 (Merrell *et al*., 2003a). This was in contrast to non-array based work which demonstrated that *vacA* was induced under acidic conditions (Barnard *et al*., 2004). Moreover, two other studies found no difference in *vacA* expression in response to acid exposure (Allan *et al*., 2001, Ang *et al*., 2001). RNA dot blot analysis revealed a significant increase in the accumulation of *vacA* transcripts under iron-restricted conditions (Szczebara *et al*., 1999). However transcriptional regulation of *vacA* is Fur independent (Szczebara *et al*., 1999).

vacA expression has been found to be growth phase dependent. Microarray studies and RNA dot blots have shown that *vacA* transcription appears to peak at late log to early stationary phase when strains are cultured *in vitro* (Thompson *et al*., 2003, Narayanan, 2005, Boonjakuakul *et al*., 2004). An increase in *vacA* transcript levels has also been observed during infection of PMNs (Gieseler *et al*., 2005). These expression patterns were also dependent on time (Gieseler *et al*., 2005). In a study by Boonjakuakul *et al.,* (2005) *vacA* levels during *H. pylori* infection of rhesus macaques were higher one week after infection than 4-6 weeks after infection (Boonjakuakul *et al*., 2005) indicating that VacA may play a role in the early stages of colonisation.

Several studies have shown that *vacA* transcript levels are increased by attachment to epithelial cells (van Amsterdam *et al*., 2003, Bach *et al*., 2002, Graham *et al*., 2002, Salama *et al*., 2000, Gieseler *et al*., 2005). Similar results were also obtained during infection of polymorphonuclear leukocytes in which 2 out of 8 strains produced higher levels of *vacA* mRNA. *vacA* was also found to be one of the most highly expressed putative virulence factors in *H. pylori* positive human gastric biopsy specimens out of a total of 18 virulence factors assessed (Boonjakuakul *et al*., 2004).

1.5.3 Other genetic factors potentially affecting VacA levels

In addition to differences in transcription, heterogeneity of VacA levels could be attributed to differences between strains in signal peptide cleavage between

vacA type s1 and s2 strains, although the effect of these differences in determining VacA levels is yet to be determined.

The passenger domains of many autotransporter proteins contain a pair of cysteine residues. These are usually found in the same location and have the same spacing. Letley *et al*., 2006 showed that these paired cysteine residues are required for high levels of VacA production.

As protein stability will influence the steady-state levels of VacA observed, the stability of VacA is important when investigating differences in the amount of VacA produced between strains, as stability may affect the quantification of VacA produced. Although there is no published data on the stability of VacA, preliminary results show that VacA is stable in broth culture supernatant over a period of 72 hours and is not degraded by bacterial cell factors (Narayanan, 2005).

1.6 Aims and objectives

The aim of this study was to investigate the effect of naturally occurring differences between *H. pylori* strains in the promoter, the 5' untranslated region and the signal peptide processing site of VacA on VacA production. The association between the level of VacA production and disease in humans was also assessed. These aims will be discussed in further detail at the beginning of each of the results chapters.

Chapter 2

2 Materials and Methods

2.1 Media

2.1.1 Luria-Bertani medium

Luria-Bertani (LB) medium was prepared using 10g of tryptone (Oxoid, UK), 10g of sodium chloride (Sigma-Aldrich, UK) and 5g of Yeast extract (Oxoid, UK), made up to 1L in distilled water. The medium was adjusted to pH 7.5 with NaOH. All media sterilised by autoclaving was heated to 121˚C at a pressure of 15psi for 15 minutes.

2.1.2 Luria-Bertani agar

Agar (Oxoid, UK) was added to LB medium to a final concentration of 1.5% w/v; then sterilised by autoclaving.

2.1.3 Brain heart infusion medium

Brain heart infusion (BHI) medium (Oxoid, UK) was prepared according to the manufacturer's guidelines (37g/L in dH_2O). BHI was sterilised by autoclaving. BHI broth was supplemented with either 0.2% (w/v) β cyclodextrin (Sigma-Aldrich, UK) added prior to autoclaving, or 5% (v/v) filter sterilised foetal bovine serum (FBS) (Invitrogen, UK) added after sterilisation.

2.1.4 Sulphite free brucella broth

Sulphite free brucella broth (SFBB) comprised 1% (w/v) tryptone (Oxoid, UK), 1% (w/v) Protease peptone (BD, France), 0.1% (w/v) Glucose (Sigma-Aldrich, UK), 0.2% (w/v) Yeast extract (Oxoid, UK), 0.5% (w/v) NaCl (Sigma-Aldrich, UK) in distilled water. SFBB was sterilised by autoclaving. SFBB was supplemented with either 0.2% (w/v) β-cyclodextrin added prior to autoclaving, or 5% (v/v) filter sterilised FBS, added after sterilisation.

2.1.5 Iso-Sensitest medium

Iso-Sensitest broth (Oxoid, UK) was prepared using 23.4g of Iso-Sensitest media per 1L of distilled water. Iso-Sensitest broth was sterilised by autoclaving.

2.1.6 Terrific broth

12g Tryptone (Oxoid, UK), 24g yeast extract and 4ml glycerol were added to 800ml of dH_2O and sterilized by autoclaving. When cool the volume was adjusted to a final volume of 1L with 100ml of filter sterilized 0.17M KH_2PO_4 and 0.72M K_2HPO_4 solution.

2.1.7 Blood agar

H. pylori were maintained on Columbia blood agar plates containing (Oxoid, UK). Alternatively blood agar plates were prepared using blood agar base # 2

(Oxoid, UK) to a final concentration of 4% (w/v) in distilled water, then sterilised by autoclaving. When cooled to less than 50 °C, 7 % (v/v) of lysed horse blood (Oxoid, UK) was added.

2.1.8 Antibiotics

If required appropriate antibiotics were then added to a final concentration of 30μg/ml for ampicillin and chloramphenicol (All Sigma-Aldrich, UK).

2.2 Culture conditions

2.2.1 *E. coli* culture on LB agar plates

E. coli strains were maintained on LB agar plates, supplemented with antibiotics as required, and incubated at 37 °C overnight.

2.2.2 *E. coli* liquid culture

Broth cultures of *E. coli* were grown in LB or Terrific broth, supplemented with antibiotics if required at a concentration of 30µg/ml, at 37°C with agitation at 200 rpm. Profiles of *E. coli* growth were monitored by optical density at 600 nm (OD_{600}).

2.2.3 *H. pylori* culture on blood agar plates

H. pylori strains were cultured on Columbia blood agar plates containing 5% horse blood (Oxoid, UK). Strains were incubated for 48h in a Microaerophilic

workstation (MACS VA500 (DW Scientific)) at 37˚C in an atmosphere of 5% carbon dioxide in a humidified atmosphere.

2.2.4 Isolation of clinical strains from gastric biopsies

Gastric biopsies from the corpus taken at endoscopy were immediately transferred to 200µl of Iso-Sensitest broth (Oxoid, UK) containing 15% (v/v) sterile glycerol. Biopsies were then swabbed over *H. pylori* strains were isolated from gastric biopsies on Columbia blood agar plates containing DENT *H. pylori* selective supplement (Oxoid, UK) and incubated for 3 days in a Microaerophilic workstation (MACS VA500 (DW Scientific)) at a temperature of 37˚C in an atmosphere of 5% carbon dioxide in a humidified atmosphere. After 3 days single colony isolates were passaged onto fresh Columbia blood agar plates containing 5% horse blood and grown in a Microaerophilic workstation for 48 hours. Frozen stocks of *H. pylori* strains were made by harvesting the growth from blood agar plates in 800µl of Iso-Sensitest medium and 15% (v/v) sterile glycerol (Courtin and Warner Ltd). Frozen stocks were stored at -80˚C.

2.2.5 *H. pylori* Broth cultures

H. pylori strains were grown on Columbia blood agar plates for 24 hours at 37˚C in a humidified atmosphere containing 5% CO_2. Growth from two plates was resuspended in 5ml SFBB containing 5% (v/v) fetal bovine serum (Invitrogen, UK) added after sterilisation. 200µl of this inoculum was added to

800 µl of sterile SFBB. The amount of this suspension required to give a starting OD_{600} of 0.1 in a final volume of 30ml was determined. The cultures were grown in a 75cm^3 tissue culture flask (Nunc™, Denmark) with shaking on a mini orbital shaker (Stuart Scientific) at 100 rpm in a Microaerophilic workstation (MACS VA500 (DW Scientific)). Samples were routinely grown on Columbia blood agar plates and Gram stained to confirm the presence of *H. pylori* and the absence of contamination by morphology.

2.2.6 Culture of *H. pylori* in tissue culture medium

H. pylori cells were grown on blood agar plates (Oxoid, UK) in a microaerophilic workstation and were harvested using a sterile swab and resuspended in 10 ml F-12 Ham medium (Sigma-Aldrich, UK) was supplemented with 1% L-glutamine (Sigma-Aldrich, UK) and 10% FBS (Invitrogen). The cell suspension was adjusted to a starting OD_{600} of 0.1 then incubated in a Microaerophilic workstation at 37˚C.

2.2.7 Co-culture of *H. pylori* and AGS cells

AGS cells derived from human gastric carcinoma were cultured in 25ml of F12 Ham medium with 1% L-glutamine and 10% FBS at 37˚C in an atmosphere of 5% carbon dioxide. Confluent T75 flasks of cells were trypsinised, washed and resuspended in 10ml F12 medium with l-glutamine and 10% FBS. 24-well plates were seeded with 240µl of the cell suspension added to 1ml of medium per well and incubated for 24 hours at 37˚C in an atmosphere of 5% carbon

dioxide and 80% humidity. The medium was then removed from the wells and replaced with 300µl pre warmed fresh medium. Three plates of 24h *H. pylori* growth was resuspended in 5ml sterile PBS. This bacterial suspension was adjusted to a final OD_{600} of 0.01, (2.5 x 10^6 bacteria) and added to each well of a 24 well plate. *H. pylori* cells were layered onto AGS cells at 1000 x G for 20 minutes at 20˚C in a bench top centrifuge. *H. pylori* cells that had not bound to the surface of the AGS cells were removed by washing twice with fresh media. The media was replaced and the plates incubated at 37˚C in an atmosphere of 5% carbon dioxide and 80% humidity. Cells were removed from wells by manual agitation and the cells resuspended by pipetting. In addition wells containing only *H. pylori* were used as a negative control and set up in the same way. Samples were taken and stabilized using RNA protect™ Bacteria Reagent (Qiagen, UK) prior to extraction.

2.3 Collection of gastric biopsies

Consenting patients attending upper gastrointestinal endoscopy at the Queens Medical Centre, Nottingham donated two gastric biopsies from the corpus; one of which was immediately stored in RNA later (Qiagen, UK) and the other in 200µl of sterile Iso-Sensitest broth (Oxoid, UK) containing 15% (v/v) sterile glycerol. Additional corpal and antral biopsies were taken for routine diagnostic testing, histopathology, *H. pylori* culture and isolation. CLO (Camplyobacter-like organism test) (Kimberly-Clark, USA) were carried out at the time of the endoscopy using an antral biopsy according to the manufacturer's directions. The result was used in conjunction with *H. pylori*

culture and histology to determine the *H. pylori* infection and disease status of the patient.

2.4 Strains and plasmids

H. pylori and *E. coli* strains used in this study, and their relevant characteristics are shown in Table 2.1. *H. pylori* was identified by morphology, Gram staining and urease testing. The strains used in this study were isolated and typed by myself, J. Hale, J. Rhead, S. Patel and A. Memon. Table 2.2 shows the details of the plasmids used in this study and their relevant characteristics.

Table 2.1 Bacterial strains

Strain	Relevant characteristics	Reference/Source
NovaXG Zappers™ Electrocompetent cells	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *endA1 recA1 Φ80dlacZΔM15 ΔlacX74 araD139 Δ(ara-leu)7697 galU galK rpsL nupGλ⁻ tonA*	Novagen
60190	Wild-type, VacA type s1/m1, Tox+	Leunk *et al.* (1988)
60190/A130	As 60190. except *vacA*::*km*	Cover *et al*, 1994
60190 pCTB2::*cat*	pBluescript containing 3′ *cysS* to *vacA* nt 274 from strain 60190. *cat* inserted into *Hind* III site immediately 3′ to *cysS* ; Amp^R; Cm^R	Letley *et al.* (2003)
60190 P(-6)G	As 60190 pCTB2::*cat* except proline replaced with glycine at position -6 of the signal sequence.	D.P. Letley
60190 S(-3)L	As 60190 pCTB2::*cat* except serine replaced with leucine at position -3 of the signal sequence.	D.P. Letley
60190 P(-6)GS(-3)L	As 60190 pCTB2::*cat* except proline replaced with glycine at position -6, and serine replaced with leucine at position -3 of the signal sequence.	D.P. Letley
Tx30a	Wild-type, VacA type s2/m2, Tox-	Leunk *et al.* (1988)
Tx30a pA153::*cat*	As Tx30a but *cat* inserted immediately 3′ to *cysS*; Cm^R	Letley *et al.* (2003)
Tx30a G(-6)P	As Tx30a pA153::*cat* except glycine replaced with proline at position -6 of the signal sequence.	D.P. Letley
Tx30a L(-3)S	As Tx30a pA153::*cat* except leucine replaced with serine at position -6 of the signal sequence.	D.P. Letley
Tx30a G(-6)PL(-3)S	As Tx30a pA153::*cat* except glycine replaced with proline at position -6, and leucine replaced with serine at position -3 of the signal sequence.	D.P. Letley
60190 G(-6)PL(-3)S	As Tx30a G(-6)PL(-3)S but with plasmid transformed into a 60190 strain background.	D.P. Letley and C.G. Masters (This study).
Tx30a P(-6)GS(-3)L	As 60190 P(-6)GS(-3)L but with plasmid transformed into a Tx30a strain background.	D.P. Letley and C.G. Masters (This study).
60190/ΔSL	As pCTB2::*cat* except stem loop deleted from *vacA* nt 608 to 704 and replaced with a *Bgl* II site; Amp^R; Cm^R	G.L. Narayanan
60190/SL GtoA	As 60190 pCTB2::*cat* except with G at position +28 within the *vacA* 5′ UTR of 60190 was replaced with A.	This study
60190/IRDIS	As 60190 pCTB2::*cat* except with a 6bp disruption in an inverted repeat located … bases upstream from the -35 motif of the *vacA* promoter.	This study
45B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
46B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* -.	This study
72B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
73B	Clinical strain isolated from corpus. *vacA* type s1/m2/i2, *cagA* +.	This study
77B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
83B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
86B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA +*.	This study
93B	Clinical strain isolated from corpus. *vacA* type s1/m2/i1, *cagA*	This study

120B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
121B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
122B	Clinical strain isolated from corpus. *vacA* type s1/m1/i2, *cagA* +.	This study
124B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
126B	Clinical strain isolated from corpus. *vacA* type s1/m2/i1, *cagA* +.	This study
173B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
181B	Clinical strain isolated from corpus. *vacA* type s1/m1/m2/i2, *cagA* -.	This study
182B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* -.	This study
187B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
192B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
194B	Clinical strain isolated from corpus. *vacA* type s1/m2/i1, *cagA* -.	This study
201B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
206B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
215B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
218B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
221B	Clinical strain isolated from corpus. *vacA* type s1/m2/i2, *cagA* -.	This study
223B	Clinical strain isolated from corpus. *vacA* type s2/m2/i2, *cagA* -.	This study
233B	Clinical strain isolated from corpus. *vacA* type s2/m2/i2, *cagA* +.	This study
240B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
242B	Clinical strain isolated from corpus. *vacA* type s2/m2/i2, *cagA* -.	This study
247B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
249B	Clinical strain isolated from corpus. *vacA* type s1/m2/i1/i2, *cagA* +.	This study
253B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study

255B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
256B	Clinical strain isolated from corpus. *vacA* type s2/m2/i2, *cagA* +.	This study
265B	Clinical strain isolated from corpus. *vacA* type s1/m2/i2, *cagA* +.	This study
271B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* -.	This study
274B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
308B	Clinical strain isolated from corpus. *vacA* type s1/m2/i2, *cagA* -.	This study
320B	Clinical strain isolated from corpus. *vacA* type s1/m2/i2, *cagA* -.	This study
322B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
326B	Clinical strain isolated from corpus. *vacA* type s1/i2/m2, *cagA* -.	This study
335B	Clinical strain isolated from corpus. *vacA* type s1/m1/i1, *cagA* +.	This study
350B	Clinical strain isolated from corpus. *vacA* type s1/m2/i1, *cagA* +.	This study

Table 2.2 Plasmids

Name	Construct	Resistance	Reference/Source
pCTB2::*cat*	pBluescript containing 3' *cysS* to *vacA* nt 274 from strain 60190. *cat* inserted into *Hind* III site immediately 3' to *cysS* ; Amp^R; Cm^R	Ampicillin, chloramphenicol	Letley *et al*, 2000
pCTB2::*cat* P(-6)GS(-3)L	As 60190 pCTB2::*cat* except proline replaced with glycine at position -6, and serine replaced with leucine at position -3 of the signal sequence.	Ampicillin, chloramphenicol	Letley *et al*, 2000
pCTB2::cat ΔSL	As pCTB2::cat except stem loop deleted from *vacA* nt 608 to 704 and replaced with a Bgl II site; AmpR; CmR.	Ampicillin, chloramphenicol	G.L. Narayanan 2005

2.5 Isolation, manipulation and analysis of DNA

2.5.1 Preparation of *H. pylori* genomic DNA

H. pylori was harvested from blood agar plates or broth cultures, and resuspended into 1ml of phosphate buffered saline (PBS) (Oxoid, UK) then centrifuged at 13000 rpm for 3 minutes. Cell pellets were resuspended in 100µl of TE buffer (10mM Tris-Cl, 1 mM EDTA, pH 8.0). 500µl of GES buffer (5M guanidine thiocyanate, 0.1M EDTA, 0.5% (w/v) sarkosyl) was added and left to incubate at room temperature for 5 minutes, then placed on ice. 250µl of ice cold ammonium acetate (7.5M) (Sigma-Aldrich, UK) was added, gently mixed and incubated on ice for a further 5 minutes. 650µl of chloroform was added and mixed gently. Samples were then centrifuged at 13000 rpm for 5 minutes. The upper aqueous layer was transferred to a fresh tube, and another 650µl of chloroform was added and centrifuged as before. The aqueous layer was again transferred to a fresh tube and 0.54 x volume of 100% isopropanol was added. The samples were mixed gently by inversion to allow precipitation of DNA and then centrifuged at 13000 rpm for 5 minutes. The DNA pellet was washed twice with 500µl of 70% ethanol, and allowed to dry. The pellet was resuspended in 100µl of TE buffer.

2.5.2 Natural transformation of *H. pylori*

H. pylori strains were grown on blood agar plates for 24hrs (2 plates for each transformation). Growth from 2 plates was resuspended in 900µl of iso-sensitest medium with 100µl foetal bovine serum, then centrifuged for 1

minute at 13000rpm . The supernatant was discarded and the cells were resuspended in 30µl of the iso-sensitest FBS solution. The resulting cell suspension was then placed as a small dot on the centre of a blood agar plate and incubated at 37˚C for 6 hours to allow the cells to adhere to the plate. Purified plasmid DNA was heated at 80˚C for 5 minutes to sterilise and allowed to cool. 5µl of Plasmid DNA at a concentration of 50 ng/µl was added to the cells and incubated at 37˚C overnight. The cell mass was spread over the surface of the same plate with a sterile swab and incubated for 24hrs at 37˚C the passaged on to blood agar plates containing the appropriate antibiotic to select for positive transformants.

2.5.3 Plasmid DNA extraction

Plasmids were extracted from *E. coli* liquid culture (section 2.2.2) using the QIAprep® Miniprep kit (Qiagen, UK) according to the manufacturer's recommendations.

2.5.4 Polymerase chain reaction (PCR)

Reactions were carried out in a total volume of 50µl, consisting of 5µl of 10x PCR reaction buffer (100 mM Tris-HCl, 15mM $MgCl_2$, 500mM KCl, pH 8.3 (20˚C)) (Roche, UK), 1µl of deoxynucleotide triphosphates (dNTPs) (200µM) (Promega, USA), 1µl of each of the forward and reverse primers (25µM) (see Table 2), 0.5µl of Taq polymerase (Roche, Germany), and 40.5µl of nuclease free water. PCR conditions were as shown in Table 2.3. The annealing

temperatures for the primers were calculated based on the number of purine and pyrimidine bases present, using the formula: Tm = (2 x (A+T)) + (4 x (G+C) (Wallace rule)). The extension time was based on the expected length of the product. For Taq polymerase (Roche, UK) 1 minute of extension time was allotted per kb of product.

Table 2.3 PCR conditions

Step	Number of cycles	Temperature	Length of time
1	1	95˚C	1 min 30 secs
2	35	95˚C	30 secs
		Variable annealing temperature	1 min
		72˚C	Variable extension time
3	1	72˚C	5 min

2.5.4.1 Primers

Details of the primers used in this study are shown in Table 2.4

Table 2.4 Primers

Primer Name	Primer Sequence 5' to 3'	Reference
A1043	ATTTTACCTTTTTACACACATTCTAGCC	Atherton *et al* 1999
VacR10	TTATAAGTCCCTACAGCGTC	D. L. Letley (Unpublished)
C1226	CTGCTTGAATGCGCCAAAC	Cover *et al* 1994
A3436	ATGGAAATACAACAAACACAC	Cover *et al* 1994
DL1	GCTTTGATGGACACCCCACAAGG	Avilez-Jimenez *et al* 2004
C0374	CTCCAGAACCCACACGATT	D. L. Letley (Unpublished)
Cys2F	GGATCAAAACCCTAAAAACA	D. L. Letley (Unpublished)
VagF	CAATCTGTCCAATCAAGCGAG	Cover *et al* 1994
VagR	CTAGCGTCAAAAATAATTCCAAGG	Cover *et al* 1994
VacF1	GTTGGGATTGGGGGAATGCCG	Avilez-Jimenez *et al* 2004
C1R	TTAATTTAACGCTGTTTGAAG	Rhead *et al* 2007
C2R	GATCAACGCTCTGATTTGA	Rhead *et al* 2007
16s rRNA F	CGATGAAGCTTCTAGCTTGC	Narayanan 2005
16s rRNA R	ATAGGACATAGGCTGATCTC	Narayanan 2005
Cag2	GGAACCCTAGTCGGTAATG	Rudi *et al* 1998
Cag4	ATCTTTGAGCTTGTCTATCG	Rudi *et al* 1998
VacR5	CATGCTTTGATTGCCGATAGC	Avilez-Jimenez *et al* 2004
LepB forward	TTTTATCACCCCTGTGCCT	N. C. Doherty (Unpublished)
LepB reverse	GGATTTTCATTAAGGTGG	N. C. Doherty (Unpublished)

2.5.5 Analysis of DNA by agarose gel electrophoresis

DNA fragments were separated by electrophoresis using 0.8% or 2% agarose (Seakem, UK) gels prepared in 1 x Tris-acetate – EDTA (TAE) buffer (40 mM Tris-acetate 2mM EDTA, pH 8.5), containing ethidium bromide (0.5µg/ml). Samples were loaded in 1 x loading buffer (6 x 0.25% bromophenol blue, 20% glycerol in TE, 0.1% SDS). 5µl of a 1Kb DNA ladder was used at a concentration of 0.1µg/µl (Invitrogen, UK). Gels were run at 90V in 1x TAE buffer. DNA was visualised under ultra-violet light (Uvidoc).

2.5.6 Purification of DNA fragments

PCR fragments were purified using Promega Wizard SV Gel and PCR clean-up system (Promega, UK) according to the manufacturer's recommendations.

2.5.7 DNA sequencing and analysis

DNA sequencing was performed using purified PCR fragments at the Biopolymer Synthesis and Analysis unit (BSAU) (Queen's Medical Centre, University of Nottingham). DNA sequence analysis was carried out using SeqMan and MegAlign software (DNAstar Inc).

2.5.8 Site directed mutagenesis

Reactions were carried out in a total volume of 50µl, consisting of 5µl of 10x PFU buffer (Stratagene, UK), 1µl of deoxynucleotide triphosphates (dNTPs) (200µM) (Promega, USA), 1µl of each of the forward and reverse primers (25µM) (

Table 2.5), 1µl of PFU polymerase (Stratagene, UK), and 40µl of nuclease free water. Finally 1µl of pCTB2::*cat* plasmid DNA was added at a final concentration of 1ng/ µl. PCR conditions were as shown in Table 2.6. 1µl of *Dpn1* enzyme (Stratagene) to each PCR reaction and incubated for 1.5 hours at 37˚C to digest methylated DNA.

Table 2.5 Primers used for site directed mutagenesis

Primer Name	Sequence (5' to 3')	Reference
IRDIS Forward	TCTAGCCAAAAATTCTCATAGAATTGCTTTTTAATCT	This study
IRDIS Reverse	AGATTAAAAAGCAATTCTATGAGAATTTTTGGCTAGA	This study
SL GtoA Forward	TACTGCATATTTATAACCTTAATCGTAAATG	This study
SL GtoA Reverse	CATTTACGATTAAGGTTATAAATATGCAGTA	This study

Table 2.6 PCR conditions for site directed mutagenesis

Step	Number of cycles	Temperature	Length of time
1	1	95˚C	1 min 30 secs
2	18	95˚C	1 min
		60˚C	1 min
		72˚C	15min
3	1	72˚C	7 min

2.5.9 Transformation of *E. coli* by electroporation

For each reaction 1µl of dialysed DNA solution (as highly concentrated as possible) was added to a pre chilled electroporation cuvette (0.1-cm gap). 25µl of NovaXG Zappers™ Electrocompetent cells were added to the electroporation cuvette without introducing bubbles. Cells were electroporated

at 1.8 kV. Within 10 seconds of the pulse, 975µl of room temperature Terrific broth medium was added to the cuvette, then transferred to a 5ml universal and incubated at 37°C with shaking at 225–250 rpm for 60 min prior to plating. 100 µl of cells were plated directly onto LB agar plates containing the appropriate antibiotics and incubated at 37˚C overnight. Antibiotic resistant colonies were isolated and grown in 5ml of LB supplemented with the appropriate antibiotics, at 37°C with shaking at 200 rpm overnight.

2.6 Isolation and Analysis of RNA

2.6.1 Preparation of RNA from bacterial cultures and AGS cell co-culture

Broth culture and tissue culture samples were grown as described in sections 2.4.5 and 2.4.7. The RNA Mini kit is optimized to extract RNA from no more than $2.5x10^8$ cells. 1ml of *H. pylori* culture of an OD_{600} of 1 contains approximately $1x10^9$ cells. Thus the volume of culture containing $2.5x10^8$ cells was calculated and 2 x volumes of RNA protect™ Bacteria Reagent (Qiagen, UK) were added to this volume of culture, then vortexed for 5 seconds. The stabilized broth culture was left to incubate at room temperature for 5 minutes then centrifuged at 5000g for 10 minutes. The supernatant was then decanted and any residual supernatant was removed by gently dabbing the inverted tube onto a paper towel. Cell pellets were stored at – 80˚C to minimise degradation of RNA. Total RNA was prepared using the RNeasy® Mini Kit (QIAGEN, UK). To lyse the cells, 20µl of Qiagen proteinase K (1 mg/ml) was added to

200µl DPEC treated TE buffer containing 15mg/ml lysozyme. This mixture was then added to the pellet and vortexed for 10 seconds and incubated for 10 minutes, vortexing for 10 seconds every 2 minutes during incubation. The subsequent RNA extraction was performed according to the manufacturer's protocol. RNA was eluted in 30 µl of RNase-free water was added directly to the spin column membrane, and centrifuged for 1 min at 10000 rpm to elute the RNA. This elution step was repeated with the original eluate to obtain a higher final concentration of RNA. The resulting RNA samples were stored at -80 ˚C in order to minimise RNA degradation. The resulting RNA samples were stored at -80 ˚C to minimise RNA degradation.

2.6.2 Preparation of RNA from gastric biopsies

At endoscopy, gastric biopsies for RNA extraction were transferred immediately to 250µl of RNA later stabilization reagent (Qiagen, UK) and stored at -80˚C according to the manufacturer's recommended protocol. Biopsies that had been briefly thawed on ice were transferred to 600µl of Buffer RLT and β-mercaptoethanol (Qiagen, UK), and homogenized for 20-40 seconds using a T8 ultra Turrax rotor-stator homogeniser (IKA, Werke & Co., Freiburg, Germany). The homogenized samples were added to QIAshredder columns (Qiagen, UK) and centrifuged for 2 minutes at maximum speed. The lysate was spun for 3 minutes at full speed, and the supernatant transferred to a new microfuge tube. Total RNA was prepared using the RNeasy® Mini Kit (Qiagen, UK) according the manufacturers protocol. RNA was eluted in 30 µl of RNase-free water was added directly to the spin column membrane, and

centrifuged for 1 min at 10000 rpm to elute the RNA. This elution step was repeated with the original eluate to obtain a higher final concentration of RNA. The resulting RNA samples were stored at -80˚C in order to minimise RNA degradation.

2.6.3 Quantitation of total RNA

Once the total RNA was extracted from the biopsy and the broth culture samples, the yield of total RNA obtained was assessed using the Nanodrop spectrophotometer (Nanodrop ND-1000, Nanodrop Technologies Inc., Wilmington, USA). The concentration of total RNA (expressed as ng/µl) and both the 260:280 and 260:230 ratios were measured and recorded.

2.6.4 DNA-free treatment of RNA

3µl of 10x DNaseI buffer (Ambion, Ltd) and 1µl rDNaseI (Ambion, Ltd) was added to the RNA (which was adjusted to a concentration of 33.3ng/µl in the total DNA-free reaction mix with RNase free water), and incubated at 37˚C for 30 minutes. 3µl of the resuspended DNase Inactivation Reagent (Ambion, Ltd) was then added, mixed well, and incubated at room temperature for 2 minutes vortexing the mixture 3 times during the incubation period to redisperse the DNase Inactivation Reagent. The mixture was then centrifuged at 10,000 x g for 1.5 minutes to pellet the DNase Inactivation Reagent. The supernatant containing the DNA-free treated RNA was transferred to a fresh tube and stored at -80˚C.

2.6.5 cDNA synthesis

RNA was transcribed using a QIAGEN Omniscript™ reverse transcriptase kit. Reactions were carried out in a total volume of 20µl, consisting of 2µl Buffer RT, 2µl of dNTP mix, 2µl of pd$(N)_6$ 5'-Phosphate, Sodium salt random hexamer primer (Amersham, UK), 1µl of RNase inhibitor (10U/µ), 6µl of water, 1µl of Omniscript reverse transcriptase and 6µl of RNA at a concentration of 33ng/µl. Reverse transcription was performed at 37 ˚C for 1 hour. Negative controls for each RNA sample were prepared in the same manner but excluded reverse transcriptase.

2.6.6 Quantitative real-time PCR

Primers were specific to *H. pylori* and checked using a Basic Local Alignment Search Tool (BLAST) search (www.ncbi.nlm.nih.gov/**BLAST**/*)* and can be found in Table 2.7. Any predicted secondary structures and dimer formation of the primers were assessed using premier biosoft's Netprimer algorithm: (http://www.premierbiosoft.com/netprimer/index.html). Real time PCR was performed using the Rotor-Gene 3000 real-time PCR system (Corbett Research, Australia), using the Quantitect™ SYBR Green PCR kit (QIAGEN, UK). Amplification was carried out in a reaction volume of 25µl and was set up according to the manufacturer's recommendations using 2µl of template cDNA diluted to 33.3ng/µl for biopsy sample (section 2.6.2) and 1.7 ng/µl for broth culture samples (section 2.6.1). Cycling conditions are outlined below in

Table 2.8. No template controls (NTCs) were included in each run, and melt curve analyses were performed to ensure primer specificity and the absence of contamination. Negative cDNA samples, produced in the absence of reverse transcriptase from each RNA sample were tested in parallel to ensure that products were not amplified from contaminating genomic DNA. Samples were run in triplicate and the results were analysed using the Pfaffl method of analysis (Pfaffl, 2001). Efficiencies of each PCR were calculated by serial dilution as described in the appropriate results chapters.

Table 2.7 Real-time PCR primers

Primer Name	Primer Sequence 5' to 3'	Reference
C1226	CTGCTTGAATGCGCCAAAC	Cover *et al* 1994
A3436	ATGGAAATACAACAAACACAC	Cover *et al* 1994
16s rRNA F	CGATGAAGCTTCTAGCTTGC	Narayanan 2005
16s rRNA R	ATAGGACATAGGCTGATCTC	Narayanan 2005

Table 2.8 Real-time PCR conditions

Step	Number of cycles	Temperature	Length of time
1.	1	50˚C	2 min
2.	1	95˚C	15 min
3. Cycling	35	95˚C	30 sec
		56˚C	60 sec
		72˚C	30 sec
		79˚C	15 sec
		72˚C	20 sec
4. Melt	1	72˚C to 95˚C rising by 1˚C each step	Wait for 45 sec on 1st step, then wait for 5 seconds for each step afterwards

2.7 Isolation and Analysis of Protein

2.7.1 Separation of secreted proteins by centrifugation

900µl samples of 24h broth cultures were centrifuged at 13000 rpm for 1 minute. The resulting supernatant was removed and transferred to a fresh eppendorf tube. The pellet and supernatant were stored at -20˚C.

2.7.2 Preparation of cellular proteins

Cell pellets were corrected for OD_{600} by resuspending in variable volumes of 1x SDS-sample loading buffer (50mM Tris-Cl, 100mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The pellets were then sonicated on ice with one, 10 second blast at an amplitude of 15 microns.

2.7.3 SDS-PAGE analyses of *H. pylori* proteins

Samples were boiled for 5min in 1x SDS-sample loading buffer (50mM Tris-Cl, 100mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and centrifuged for 3 min at 13000 rpm prior to loading. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were cast in Mini-protein casting trays (BioRad, USA) and comprised of a 9% polyacrylamide resolving gel (For 1 gel: 1.25ml separating buffer (1.5 M Tris-HCl pH 8.8, 0.4% (w/v) SDS), 1.5ml 30% Bis-acrylamide solution (37.5:1) (Geneflow, UK), 2.22ml water, 25µl APS 10% (w/v) (SIGMA, USA), 2.5µl TEMED (SIGMA, USA)) and a 3% stacking gel (For 1 gel: 1ml stacking gel buffer (0.5 M Tris-HCl pH 6.8, 0.4% (w/v) SDS), 200µl acrylamide solution (30%

(w/v), 0.8% (w/v) APS, 2.5µl TEMED and water). 10µl of each sample was loaded per well, in addition to 10µl of SeeBlue Plus2 prestained standard marker (Invitrogen). Proteins were separated at 20mA through the stacking gel and 40mA through the resolving gel in SDS-PAGE electrode buffer (5x stock: 0.125M Tris, 0.95M glycine, 0.4% SDS, pH 8.3).

2.7.4 Coomassie blue staining

Gels were stained with Coomassie Brilliant Blue solution (0.1% (w/v) Coomassie brilliant blue (BIO-RAD, USA), 45% (v/v) methanol, 9% (v/v) glacial acetic acid) for 1 hour and destained with 20% (v/v) methanol and 7% (v/v) glacial acetic acid overnight or until the protein bands were clearly visible. Gels were dried using DryEase™ Mini Gel Drying System (Novex, USA) and Gel-Dry™ drying solution (Invitrogen, USA).

2.7.5 Western blotting

Proteins separated by SDS-PAGE as described above were transferred to BioTrace®NT Pure nitrocellulose blotting membrane (pre-soaked in transfer buffer - 10mM Tris, 100mM glycine, 20% (v/v) methanol), using western blotting transfer apparatus (BIO-RAD, USA). The transfer was performed at 100mA for 90 minutes. After transfer, the nitrocellulose membrane was removed, and blocked in PBST-milk (PBS (Oxoid, UK), 0.5% (v/v) Tween-20 (Sigma, UK), and 5% skimmed milk powder (Oxoid, UK)) overnight, with gentle shaking at 4˚C. The PBST-milk was then discarded and the membrane was incubated in PBST-milk with primary antibody (rabbit polyclonal anti-

VacA p58 diluted to1:10,000) for 1 hour, shaking at room temperature. The membrane was washed 3 x 5 minutes in PBST (PBS containing 0.5% (v/v) Tween-20), shaking at room temperature. The membrane was incubated in a secondary antibody solution of goat anti-rabbit IgG peroxidise conjugate (Sigma, UK) in PBST (1:20,000) for 1 hour, shaking at room temperature. The secondary antibody solution was discarded and the membrane was washed in PBST as follows: 3x 5 min, 2x 15 min, 3x 5min. The blot was developed using the ECL detection system (Amersham Pharmacia Biotech, UK) according to the manufacturer's recommendations and visualised by exposure to X-ray film (Hyperfilm^{TM}, Amersham, UK).

2.7.6 Quantitation of immunoreactive protein concentrations

Protein levels detected on the western blots were quantified using a GS800 densitometer (BioRad, USA) using the comparative Quantity One densitometry software (BioRad, USA). Immunoreactive bands were selected and the peak density measured within each band. In order to account for the background level of the blot the rolling disk option was selected.

2.7.7 N-terminal protein sequencing

Proteins were separated by SDS-PAGE as described above. The gel was then soaked in [cyclohexylamino]–1propanesulphonic acid buffer (CAPS) for 20 minutes with shaking at room temperature. PVDF membrane (Amersham biosciences) was activated in 100% methanol for 10 seconds, washed in

distilled water for 5 minutes and equilibrated in CAPS buffer for 10 minutes. Proteins were transferred to the PVDF membrane for 1 hour at 100V with cooling using western blotting transfer apparatus (BIO-RAD, USA). All blotting equipment was washed thoroughly to remove all traces of glycine prior to the transfer. After transfer, the membrane was removed, rinsed with dH_2O and stained with Coomassie blue, then destained, washed, air dried and sent to Alta Biosciences, Edgbaston, UK for N-terminal sequencing.

2.7.8 VacA enzyme linked immuno-sorbent assay (ELISA)

Equal volumes of supernatant samples were added to 2x carbonate buffer (100mM Na_2CO_3, 100mM $NaHCO_3$, pH 9.8). 100µl aliquots were dispensed into 96 well micro-titre plate (Nunc-Immuno™ Modules, Nunc, Denmark) which were incubated at 4˚C overnight to allow toxin to bind. The carbonate buffer was removed and each well was blocked with 200µl PBST-BSA (PBS with 0.1% (v/v) Tween-20, 3% (w/v) BSA (SIGMA, USA)). Plates were incubated at 37˚C for 1 hour. The wells were then washed 3 times with 200µl PBST (PBS with 0.1% (v/v) Tween-20). 100µl of primary antibody in PBST-0.1% BSA (1:5000) was added to each well and incubated at 37˚C for 1 hour. The polyclonal primary antibody used was anti-VacA p58, as used for the western blots. The wells were washed 3 times in PBST. 100µl of the secondary antibody solution (goat anti-rabbit IgG, peroxidise conjugate secondary antibody (Sigma, UK) in PBST-0.1% BSA (1:1000).) was added to each well and the plate incubated at 37˚C for 1 hour. The plate was washed 5 times in PBST. 100µl of freshly prepared Sigma Fast OPD developer (Sigma, USA)

was added to each well and incubated for 10 minutes in the dark. 50µl of 2M H_2SO_4 was added to stop the reaction. Dual optical densities for each well were determined immediately using a microplate reader (Labsystems MF) set to 492nm and 540nm. Readings at 540nm were subtracted from the 492nm readings to correct for optical imperfections in the plate.

2.8 Histopathology

Two antral and two corpal biopsies samples were taken from each patient and immediately fixed in 6 – 10% formalin in 0.9% NaCl (provided by histopathology). The biopsies were embedded in paraffin wax and 4µM sections cut for histological slide preparation. Slides were stained with Hematoxylin and eosin (H&E) for quantitative assessment of inflammatory cells and Giemsa for *H. pylori* quantification. All preparation work was carried out by technicians within the Division of Histopathology, University Hospital, Nottingham. The histopathological parameters were assessed using the Sydney scoring system, (Dixon *et al*., 1996) a visual analogue scale. Biopsies were evaluated for the presence, number and distribution of mononuclear cells (lymphocytes and plasma cells) (chronic inflammation), the density of *H. pylori* colonization, the presence number and distribution of neutrophils and eosinophils (neutrophilic activity). Glandular atrophy was recorded when the gastric glands were atrophic and reduced in number and the mucosa thinned. Intestinal metaplasia was recorded when gastric epithelium appeared to have an intestinal pattern or goblet cells were detected. The criteria for normal gastric histology were: no cells to a few mononuclear cells per high-power field (HPF;

400x magnification) and none to occasional scattered neutrophils and eosinophils without tissue changes which include loss of glandular tissue and the presence of metaplastic epithelium. Sparse lymphocyte aggregates without an increase in inflammatory cells were considered normal. The Sydney system measures chronic inflammation, polymorphonuclear activity, glandular atrophy, intestinal metaplasia and *H. pylori* colonisation density on a scale ranging from 0 – normal, to 1 – mild, 2 – moderate, and 3 – severe; evaluating one feature at a time. In cases where intensity varied considerably within the same biopsy sample the different areas were averaged and the specimen scored accordingly. (See figure 3.5 for a schematic visual analogue scale for evaluating and scoring histopathological parameters and figure 3.6 to 3.16. for photographic examples (photographs prepared by Dr Abed Zaitoun)). Other parameters such as presence of lymphoid follicles, loss of mucus layer, follicular hyperplasia and whether *H. pylori* colonisation was predominantly on the surface of epithelial cells or in the lumen were also noted. Scoring was blinded to *vacA* mRNA and strain typing results and was carried out simultaneously by me, Sapna Patel and Dr Abed Zaitoun (Division of Histopathology, University Hospital, Nottingham). Two sections from 2 corporal biopsies were examined and a score was given when all parties were in agreement.

2.9 Statistical analysis

Statistical analyses were carried out using MINITAB Release 14 and Microsoft Excel. In all cases, a significant difference or correlation was taken at $P \leq 0.05$.

Specific statistical analyses are described in more detail in each of the results chapters where appropriate.

Chapter 3

3 Investigation into associations between levels of *vacA* transcription *in vivo* and disease.

3.1 Introduction

H. pylori infection is a known risk factor for distal adenocarcinoma which is known to be induced by chronic gastric inflammation. Two histologically distinct types of gastric adenocarcinoma exist (Lauren, 1965): the diffuse type which is preceded by gastritis throughout the stomach but not necessarily with gastric atrophy, and the intestinal type which is preceded by a corporal predominant gastritis with gastric atrophy and intestinal metaplasia (Reviewed in (Atherton *et al*., 1997). The intestinal type of adenocarcinoma progresses through a cascade of histological steps which are initiated by chronic superficial gastritis leading to atrophic gastritis and intestinal metaplasia followed by dysplasia and gastric adenocarcinoma (Sipponen and Correa, 2002, Correa, 1996). Although almost all individuals infected with *H. pylori* develop gastritis only a small minority go on to develop gastric cancer. Several other risk factors are important in determining disease outcome, including host genetics, inflammatory response; environmental factors such as diet and smoking and bacterial strain specific virulence factors.

An important *H. pylori* virulence factor linked with gastric cancer is the vacuolating cytotoxin. The gene *vacA* that encodes the vacuolating cytotoxin is present in all *H. pylori* strains, but an active toxin is not produced in all cases (Cover *et al*., 1994). The *vacA* gene is generally well conserved but there is

significant allelic diversity in three regions of the gene: the signal region (s), the mid region (m) and the intermediate region (i). Characterisation of these regions has revealed that different specific forms of these regions exist (Atherton *et al*., 1995, van Doorn *et al*., 1998). This allelic diversity has allowed strains to be genotypically differentiated.

The signal region can be one of two main types; s1 or s2. Among the type s1 strain subtypes, s1a, s1b and s1c have been identified (although type s1c is restricted to Asia). Type s2 VacA is cleaved at a different site in the signal peptide sequence to type s1 resulting in a form of VacA which has a 12 amino acid hydrophilic N-terminal extension whereas mature type s1 VacA has a hydrophobic N-terminus (Atherton *et al*., 1995, Letley and Atherton, 2000). This 12 residue extension to VacA has been shown to be responsible for the loss of vacuolation induction by s2 strains (Letley *et al*., 2003, Letley and Atherton, 2000, McClain *et al*., 2001).

Within the *vacA* mid region, two main types (m1 and m2) have been identified (Atherton *et al*., 1995). The mid region has been shown to be associated with cell specificity (Atherton *et al*., 1995, Pagliaccia *et al*., 1998, Letley *et al*., 2003, Ji *et al*., 2000). m1 forms of VacA vacuolate a variety of cell types but m2 forms are more limited in the cells they vacuolate (e.g. RK13 and primary gastric cells.). Natural mosiacism occurs so that all combinations of signal and mid region types exist (Atherton *et al*., 1995). However strains with the *vacA* type s2/m1 are rarely found (Letley *et al*., 1999).

The recently identified intermediate region can also be divided into two types (i1 and i2) (Rhead *et al*., 2007). This region is located between the signal and mid regions of *vacA*. Rhead *et al* (2007) observed that *vacA* s1/m1 type strains were nearly always type-i1 and that type s2/m2 strains were always type-i2. The s1/m2 strains varied in their intermediate region type however: strains that were s1/i1/m2 showed vacuolating activity whereas type s1/i2/m2 strains were non-vacuolating. Using site directed mutagenesis they confirmed that i region type determines vacuolating activity in s1/m2 strains and that the i region influenced cell specificity in s1/m1 strains. Thus, the i region may be involved in cytotoxin binding (Rhead *et al*., 2007).

The particular *vacA* genotype of an infecting strain determines toxin production (Israel *et al*., 2001), and is a marker for the pathogenicity of an individual strain (Atherton *et al*., 1995, Rhead *et al*., 2007). Several studies have investigated the association of *vacA* type with disease outcome. In Western populations such as the USA and Western Europe, where *vacA* allelic diversity is common, *vacA* s1 genotypes are more frequently associated with higher levels of inflammation in the gastric mucosa than s2 types (Atherton *et al*., 1995, Atherton *et al*., 1997, Evans *et al*., 1998, Kidd *et al*., 1999, van Doorn *et al*., 1998). *vacA* s1/m1 and s1/m2 strains have been shown to be associated with peptic ulceration and s1/m1 type strains have been associated with gastric carcinoma (Atherton *et al*., 1995, Atherton *et al*., 1997, Rhead *et al*., 2007, Basso *et al*., 2008). Corpus neutrophil infiltration was found to be more severe in patients infected with an m1 type strain than those infected with an m2 type strain (Umit *et al*., 2009). The *vacA* i1 type is a risk factor for peptic ulcer

disease and has been shown to be associated with duodenal ulcer disease as well as gastric cancer (Basso *et al*., 2008, Rhead *et al*., 2007). Importantly Rhead *et al.* 2007 found that although *vacA* s1, i1 and m1 alleles, along with *cagA* positive status were all associated with gastric adenocarcinoma in an Iranian population, the association was greatest with i1-type strains. Furthermore, only i region status was independent of all other alleles studied. They concluded that the *vacA* i region is the best independent marker of toxicity and pathogenicity of a strain, and that i region type may be sufficient for identifying all pathogenic forms of *vacA* (Rhead *et al*., 2007).

In East Asian and Southeast Asian countries, the association between *vacA* allelic diversity and clinical outcome is not well established as the *vacA* allelic type is quite homogeneous, with the majority of the population being infected with type s1/i1 strains (Maeda *et al*., 1998, Yamaoaka *et al*., 1998). However, it is possible that a high prevalence of the more virulent *vacA* s1/i1 type strains may contribute to the high incidence of gastric cancer in this region of the world (Rhead *et al*., 2007). However, not all individuals infected with more virulent strains go on to develop disease.

Heterogeneity in *vacA* transcription between strains is known to exist (Forsyth *et al.*, 1998). In addition to differences in toxicity, production of the vacuolating cytotoxin is related to the mosaic structure of *vacA*, with type s1/m1 and s1/m2 strains producing on average higher levels of toxin than s2/m2 strains (Forsyth *et al*., 1998). VacA amount as well as the type and activity is thought to have important implications for disease; large amounts of

purified VacA induce ulcer-like erosions in mice, and VacA increases gastric ulcer formation in experimentally infected gerbils (Ghiara *et al*., 1995, Ogura *et al*., 2000). Orally administered toxigenic *H. pylori* sonicates and purified VacA also induce epithelial vacuolation, loss of gastric gland architecture (Ghiara *et al*., 1995, Telford *et al*., 1994a, Supajatura *et al*., 2002) and infiltration of mononuclear cells into the lamina propria (Telford *et al*., 1994, Supajatura *et al*., 2002). Furthermore, a small study carried out by G. L. Narayanan (2005) showed that there was an association between the occurrence of ulcers or erosions in the patient and a high level of *vacA* transcript produced by an infecting strain *in vivo*. Heterogeneity in *vacA* transcription levels between *H. pylori* strains may be a factor that contributes to different vacuolating cytotoxin phenotypes.

3.1.1 Aims

The aims of this study were to:

- Determine the level of *vacA* transcript produced per *H. pylori* cell in corporal gastric biopsies taken from *H. pylori* infected patients.
- Assess the association between the level of *vacA* expression of an infecting strain *in vivo* and the level of chronic inflammation, neutrophilic activity, the level of *H. pylori* density and the presence of glandular atrophy, and intestinal metaplasia in the infected patient.
- To determine whether the *vacA* transcript level of an infecting strain *in vivo* could explain the imperfect correlation between *vacA* type and disease.

3.2 Results

3.2.1 Quantitative real-time PCR showed large differences in *vacA* transcription between strains

Gastric biopsy samples were taken from 39 consenting *H. pylori* positive patients (determined by both culture and CLO test). Two gastric biopsies were taken from the corpus during routine upper GI endoscopy under informed consent. One of which was immediately placed in RNA*later*™ (QIAGEN) in the clinic to provide a snapshot of the mRNA present with limited interference from handling then stored according the manufacturer's instructions at -80˚C. The other biopsy was stored in 200µl of sterile Iso-Sensitest broth (Oxoid, UK) with 15% (v/v) sterile glycerol for *H. pylori* culture. Additional corporal biopsies were taken for histological examination and were fixed in 10% buffered formalin solution. Patients were normally referred for endoscopy to investigate symptoms such as dyspepsia and chronic indigestion but were otherwise healthy. Disease severity and other observations from the endoscopy were noted alongside the patients' symptoms. Samples were not taken from patients who had taken antibiotics or proton pump inhibitors two weeks prior to the endoscopy. Patients ranged in age from 29 to 81 years, 51% were male and 49% were female. The male and female groups had median ages of 49 years and 62 years respectively.

In order to quantify the amount of *vacA* transcript present per *H. pylori* cell, in corporal gastric biopsies, RNA was extracted from the gastric biopsies as described in section 2.6.2. The quality of the purified RNA is critical for accurate mRNA quantification by real-time PCR. Total RNA was quantified

using the Nanodrop to check for protein and organic contamination samples; 260/280 and 260/230 ratios of less than 1.8 were not used for subsequent analysis. RNA samples were then adjusted to 33.3ng/µl to adjust for biopsy size and any DNA contamination was removed using a DNAfree kit (Ambion) as described in section 2.6.4. cDNA was synthesised using 1µg of total RNA using the Omniscript RT kit (Qiagen, UK) as described in section 2.6.5 which included an RNase inhibitor, as RNases carried over from the RNA isolation process can affect the apparent reverse transcription efficiency (Freeman *et al*., 1999, Schwabe *et al*., 2000). A "minus reverse transcriptase" mix containing the cDNA master mix and RNA sample without reverse transcriptase was also produced for each sample to confirm the absence of residual DNA in the RNA samples. A cDNA water blank was also produced which contained the cDNA master mix and water to confirm the absence of contamination in the master mix.

Quantitative real-time PCR was performed on the Rotorgene 3000 using the methodology outlined in section 2.6.6 using the cycling conditions detailed in table 2.8. The specificity of the primers was checked using a BLAST search. The results show that the primers are specific to the genes of interest and would not amplify product from any other species residing in the gastric mucosa. Primers were chosen for their lack of predicted secondary structure and dimer formation. Predictions were carried out using Netprimer (Premier Biosoft) http://www.premierbiosoft.com/netprimer/index.html.

Differences in *vacA* mRNA levels between patients were assessed using relative quantitation which determines differences in mRNA levels across multiple samples and expresses it relative to that of a control sample which is included on every run. The level of expression of a gene is based on the distinct cycle that the fluorescence of a sample crosses a threshold level of fluorescence which is selected by the user at a level where amplification is exponential. For accuracy the threshold needs to be set as low as possible and needs to be the same on each real-time run. In this study the threshold was set at 0.01.

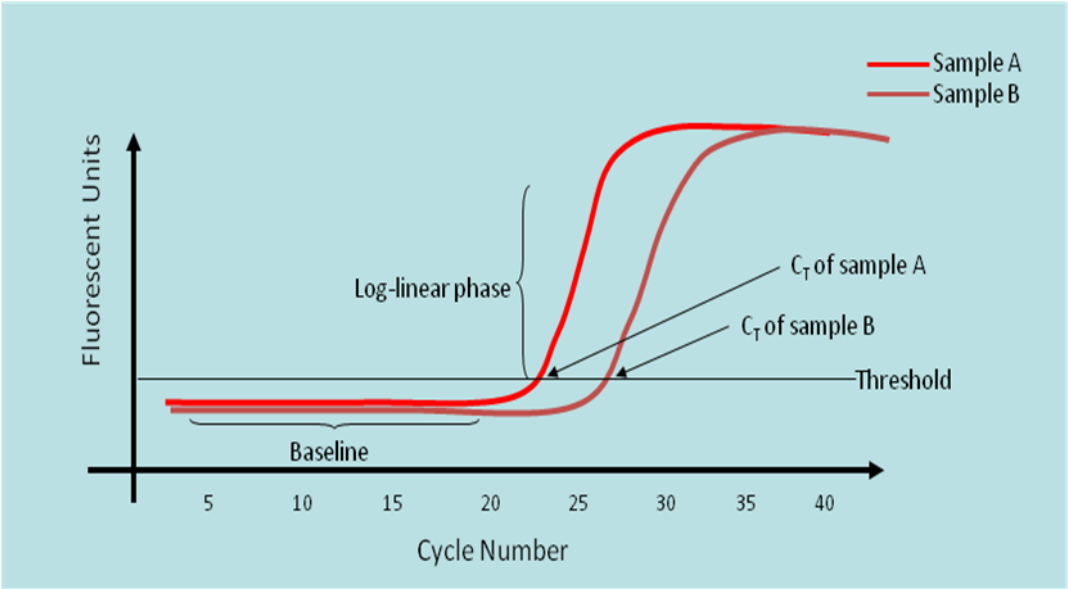


Figure 3.1Amplification plots produced by real-time PCR machines (diagram adapted from original in Narayanan 2005). The above diagram represents a typical example of an amplification plot produced as a result of a quantitative real-time PCR reaction. The graph shows that sample A has a higher concentration of starting template than sample B, as the level of fluorescence for this sample is detected after fewer cycles than sample B. The early part of the curves are represented by the baseline which represents noise at the start of the PCR. There is no detectable increase in PCR products emitting fluorescence. The threshold is adjusted by the user and represents the amount of expression above which a sample is deemed to have reacted. When setting a threshold manually, it is set in the exponential phase of the experiment, significantly above the background level to avoid noise. The cycle threshold (CT) represents the cycle number at which an increase in fluorescence is crosses the threshold which is set at the lowest level of fluorescence when the amplification of the sample is exponential. For ease of comparison the threshold was set to 0.01 in the experiments presented here.

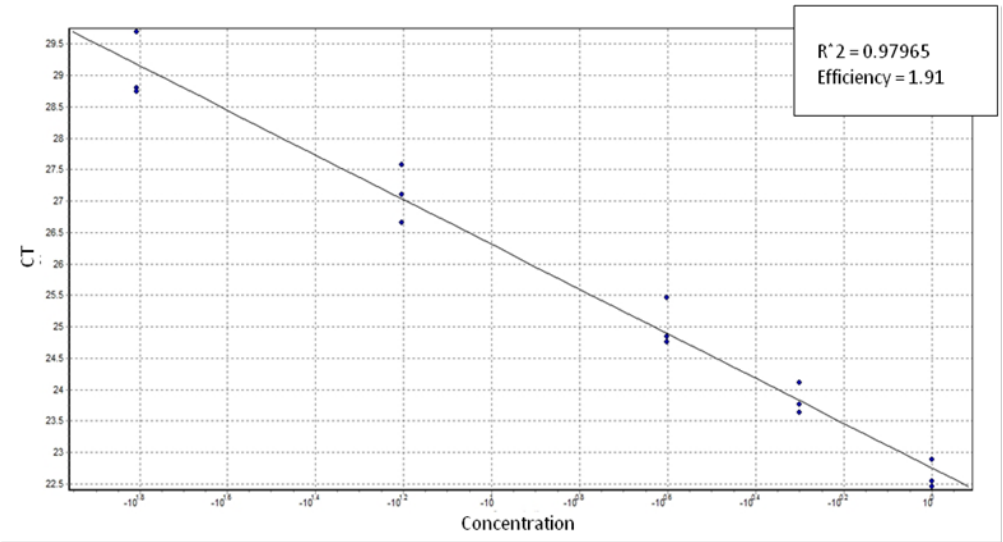
The Pfaffl method of analysis was used which calculates the expression of a target in this case *vacA* based on the difference in C_T value of the test sample (biopsy) versus the control sample (biopsy 173B) and takes into account the amplification efficiency of the gene of interest in a particular sample type. This is then adjusted to the level of expression of a housekeeping gene; in this study *16S* rRNA was used.

To determine the amplification efficiency of both *vacA* and *16S* rRNA, a sample of control cDNA was serially diluted (in triplicate) to produce a standard curve. The amplification efficiencies were calculated from the slopes of the standard curve according to the equation E = $10^{[10-1/slope]}$. The amplification efficiencies for *vacA* and *16S* rRNA were 1.91 and 1.57, respectively, with R^2 values of 0.98 and 0.99, respectively.

The optimised real-time PCR was performed in triplicate for each biopsy tested. The mean C_T value was used to calculate the relative expression. In this study, biopsy cDNA taken from patient 173B was used as the control and included in triplicate in each real-time PCR run to control for inter-experimental variations. The level of *vacA* mRNA in a biopsy was expressed relative to sample 173B the *vacA* mRNA level in this sample was taken to be 100. Blanks containing the real-time PCR master mix for both the *vacA* and *16S* rRNA. Primers were tested on each run to confirm the absence of contamination in the master mix. Melt curves for all samples tested, including the cDNA negative and blanks were checked on every run to confirm the

absence of contamination (see Figure 3.3). Melt curves are used to determine the melting temperature of the product at the end of the amplification reactions. The temperature is raised by a fraction of a degree and the change in fluorescence is measured. When the product reaches its melting temperature the two strands of DNA separate and the fluorescence rapidly decreases. The Rotorgene software plots the rate of change of the relative fluorescence units (RFU) with time (T) (d-(RFU)/dT) on the Y axis against the temperature on the x axis, which peaks at the melting temperature. All products for a particular primer pair should have similar melting temperatures unless there are contamination, mispriming or primer-dimer artefacts.

A



B

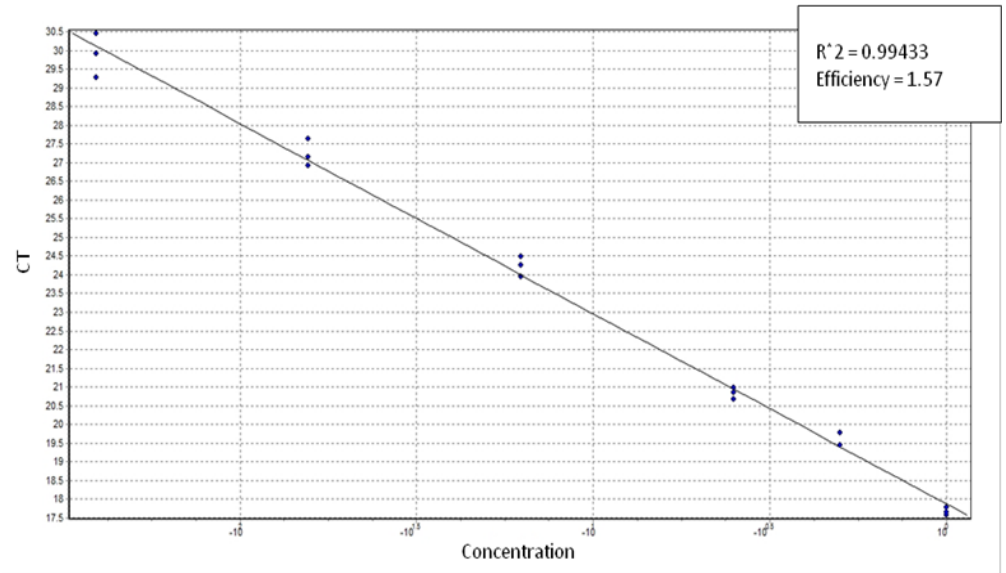
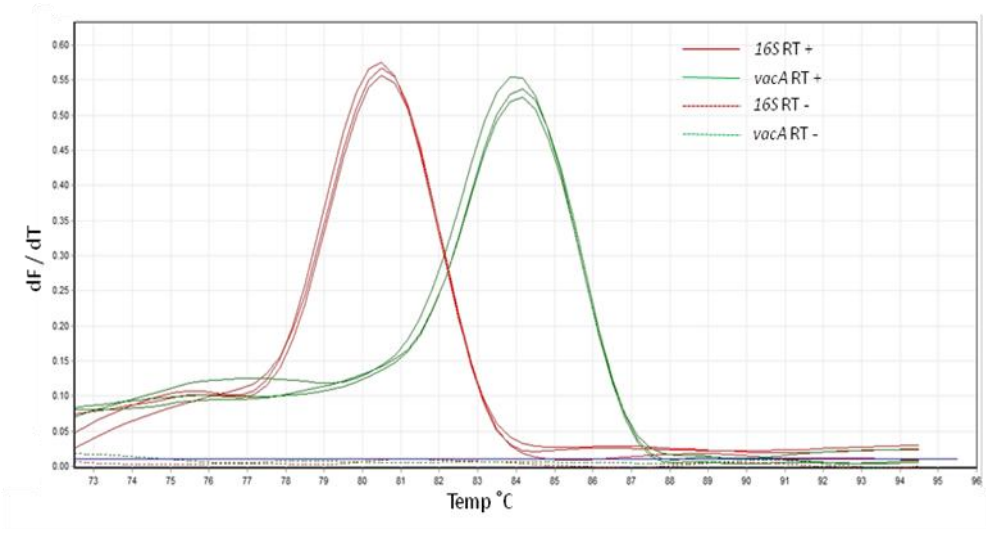


Figure 3.2 Standard curve and amplification efficiency of *vacA* (A) and 16S (B). To determine the amplification efficiencies of 16S and *vacA* using templates extracted from gastric biopsies, a sample of control cDNA 173B was serially diluted to produce a standard curve. The amplification efficiencies were calculated from the slopes of the standard curve according to the equation E = $10^{[10-1/slope]}$. The amplification efficiencies for *vacA* and 16S rRNA were 1.91 and 1.57 respectively, with R2 values of 0.979 and 0.994 respectively.

A



B

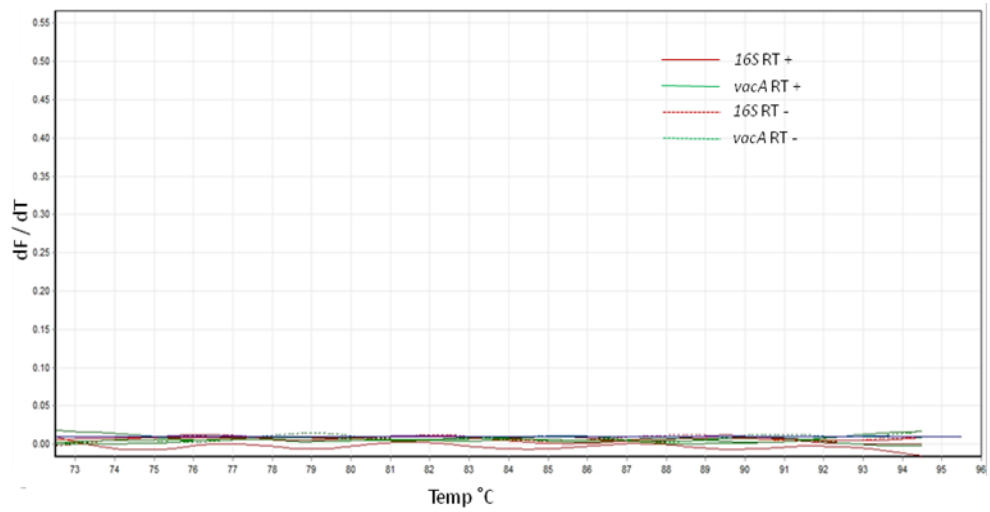


Figure 3.3 Melt curves of 16S and *vacA* PCR products synthesised from template extracted from a *H. pylori* positive biopsy (A) and a *H. pylori* negative biopsy (B). The melt curve shows the rate of change of relative fluorescence units (RFU) with time (T) (d-(RFU)/dT) on the y axis against the temperature on the x axis. The melt curves are performed at the end of the amplification reactions to determine the melting temperature of the PCR product. The single peak in each melt curve in figure 3.3 (A) indicates that only one product is present for each primer pair. The lack of peak in the RT – reactions also indicates that no contamination or primer dimer artefacts were present. The lack of peak in both the positive and negative RT reactions for primer pairs for 16S and *vacA* indicated that amplification of non-specific temples did not occur. It also indicates that no contamination or primer dimer artefacts were present.

The results show that there is a 468 fold difference in detectable *vacA* mRNA levels produced by a strain in the biopsy. The results indicate that there is a large variation in *vacA* expression amongst strains.

vacA transcript was not detectable in some of the biopsies tested. To determine the limit of detection of *vacA* mRNA using the real-time qPCR, cDNA synthesised from sample 173B that was used as a control on every run was serially diluted. The lowest relative detectable level of *vacA* was calculated using the formula: *vacA* efficiency $^{\Delta C_T (C_T \text{ 173B neat} - C_T \text{ 173B diluted})}$. Where C_T 173B neat is the C_T value of the undiluted control cDNA and C_T 173B diluted is the C_T value of the lowest dilution of the control cDNA that could be detected when the threshold was set at 0.01. The amplification efficiency of *vacA* in the biopsy cDNA was determined using the *vacA* standard curve (see Figure 3.2) and was found to be 1.91. The lowest detectable level of *vacA* was 0.18, which is 8.2 times lower than that of sample 223 which had the lowest detectable level of *vacA* in this study. Samples that had no detectable level of *vacA* transcript were considered to be non transcribers *in vivo* and were given a value of zero (see Table 3.1).

Strain	*vacA* per *16S*rRNA Titre	*vacA* Type	*cagA* Status
326	46.80	s1/m2/i2	Negative
335	35.29	s1/m1/i1	Positive
194	30.22	s1/m2/i1	Negative
187	24.65	s1/m1/i1	Positive
242	21.67	s2/m2/i2	Negative
215	9.18	s1/m1/i1	Positive
126	7.92	s1/m2/i1	Positive
271	7.86	s1/m1/i1	Negative
206	6.01	s1/m1/i1	Positive
322	5.16	s1/m1/i1	Positive
255	5.11	s1/m1/i1	Positive
247	4.14	s1/m1/i1	Positive
249	3.84	s1/m2/i1/i2	Positive
274	3.34	s1/m1/i1	Positive
265	3.26	s1/m2/i2	Positive
350	3.21	s1/m2/i1	Positive
256	1.90	s2/m2/i2	Positive
320	1.82	s1/m2/i2	Negative
253	1.52	s1/m1/i1	Positive
240	0.93	s1/m1/i1	Positive
223	0.10	s2/m2/i2	Negative
83	0	s1/m1/i1	Positive
233	0	s2/m2/i2	Positive
73	0	s1/m2/i2	Positive
121	0	s1/m1/i1	Positive
218	0	s1/m1/i1	Positive
192	0	s1/m1/i1	Positive
201	0	s1/m1/i1	Positive
221	0	s1/m2/i2	Negative
124	0	s1/m1/i1	Positive
181	0	s1/m1/m2/i2	Negative
86	0	s1/m1/i1	Positive
45	0	s1/m1/i1	Positive
72	0	s1/m1/i1	Positive
182	0	s1/m1/i1	Negative
122	0	s1/m1/i2	Positive
46	0	s1/m1/i1	Negative
77	0	s1/m1/i1	Positive
120	0	s1/m1/i1	Positive

Table 3.1 Details of strain genotypes and transcript levels within the gastric biopsies.

3.2.2 *vacA* and *cagA* genotyping of the infecting strains

To determine whether the *vacA* type and *cagA* status of the infecting strains were associated with the disease status of patients, all strains were *vacA* and *cagA* typed. To do this; strains were isolated and cultured from the second corporal biopsies as detailed in section 2.4.4 Genomic DNA was extracted from *H. pylori* strains using the methodology detailed in section 2.5.1. The *vacA* signal region was typed using A3436 and C1226 primers (Figure 3.4). The *vacA* mid region was typed using VAGF and VAGR primers (Figure 3.4). The intermediate region was typed using VacF1 and C1R for i1 type strains (Figure 3.4) and VacF1 and C2R for i2 type strains (Figure 3.4). The *cagA* status of the infecting strain was also determined using PCR typing using Cag2 and Cag4 primers (Figure 3.4). All typing PCRs were carried out using the cycling conditions detailed in section 2.5.4 and run on a 2% agarose gel. 32 strains were typed by me; the other 7 strains were typed by Sapna Patel and James Hale. Examples of the *vacA* and *cagA* typing gels are shown in Figure 3.4 and the *vacA* type and *cagA* status of all of the infecting strains are shown in Table 3.1.

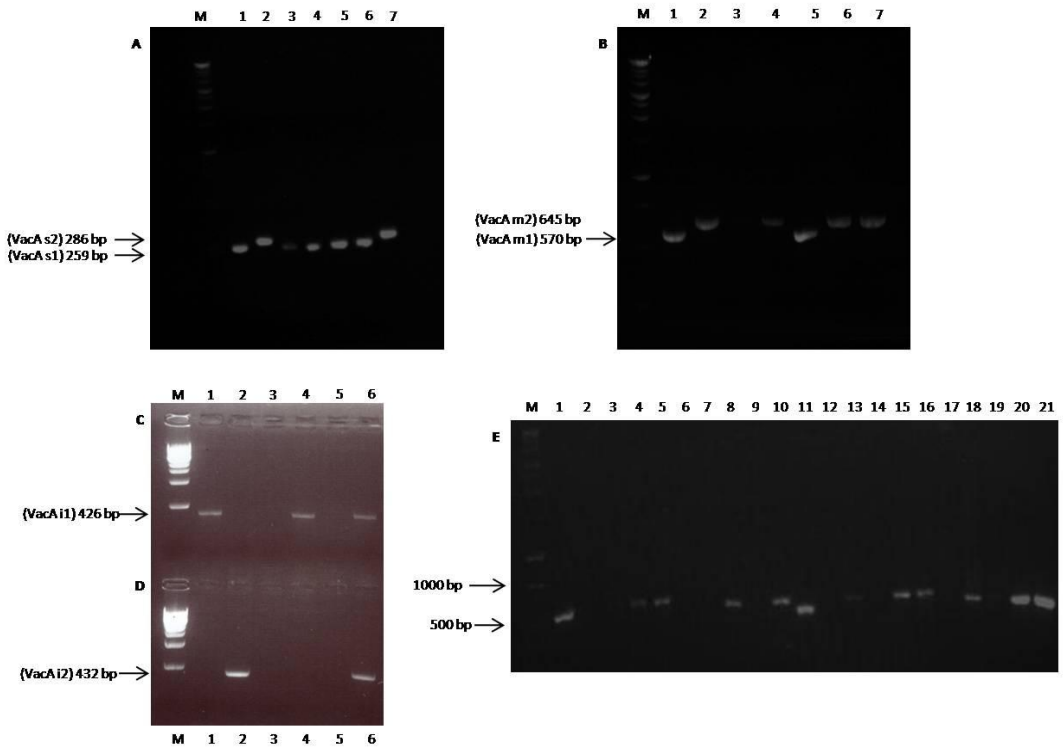


Figure 3.4 shows 2% agarose gels containing PCR products from *vacA* and *cagA* typing PCRs. Gel (A) contains PCR products amplified using primers A3436 and C1226 which amplify the *vacA* signal region, a product of 259 bp indicates the presence of *vacA* of the s1 type whereas a product of 286 bp indicates the presence of *vacA* of the s2 type. Gel (B) contains PCR products amplified using primers VAGF and VAGR which amplify the *vacA* mid region, a product of 570 bp indicates the presence of *vacA* of the m1 type whereas a product of 645 bp indicates the presence of *vacA* of the m2 type. Gel (C) contains PCR products amplified using primers VacF1 and C1R which amplify the *vacA* intermediate region of strains that possess the i1 allelic form of this toxin, a product of 426 bp indicates the presence of *vacA* of the i1 type. Gel (D) contains PCR products amplified using primers VacF1 and C2R which amplify the *vacA* intermediate region of strains that possess the i2 allelic form of this toxin, a product of 432 bp indicates the presence of *vacA* of the i2 type. Gel (E) contains PCR products amplified using primers Cag2 and Cag4 which amplify the *cagA* variable region. A PCR product between 500 bp and 750 bp is visible for strains that posses the *cagA* pathogenicity island. Lane M contains a 1kb DNA ladder in all 5 typing gels.

3.2.3 Relationships between *vacA* mRNA level and histological severity

The relationship between the *vacA* mRNA level of an infecting strain and the histological severity in the corpus of the patient was investigated. To do this, corporal biopsies taken from thirty nine *H. pylori* positive patients were fixed in 6 – 10% formalin in 0.9% NaCl. The biopsies were paraffin wax-embedded, cut into 4μm sections, and stained with hematoxylin and eosin (H&E) for quantitative assessment of inflammatory cells, Giemsa for *H. pylori* quantification and Alcian blue for confirmation of intestinal metaplasia. All preparation work was carried out by technicians under the supervision of Dr Abed Zaitoun (Division of Histopathology, University Hospital, Nottingham). The histopathological parameters were assessed using the Sydney scoring system, (Dixon *et al*., 1996) a visual analogue scale. Biopsies were evaluated for the presence, number and distribution of mononuclear cells (lymphocytes and plasma cells) (Chronic inflammation), the density of *H. pylori* colonization, the presence number and distribution of neutrophils and eosinophils (neutrophilic activity). Glandular atrophy was recorded when the gastric glands were atrophic and reduced in number and the mucosa thinned. Intestinal metaplasia was recorded when gastric epithelium appeared to have an intestinal pattern or goblet cells were detected. The criteria for normal gastric histology were: no cells to a few mononuclear cells per high-power field (HPF; 400 x magnification) and none to occasional scattered neutrophils and eosinophils without tissue changes which include loss of glandular tissue and the presence of metaplastic epithelium. Sparse lymphocyte aggregates without

an increase in inflammatory cells were considered normal. The Sydney system measures chronic inflammation, polymorphonuclear activity, glandular atrophy, intestinal metaplasia and *H. pylori* colonisation density on a scale ranging from 0 – normal, to 1 – mild, 2 – moderate, and 3 – severe; evaluating one feature at a time. In cases where intensity varied considerably within the same biopsy sample the different areas were averaged and the specimen scored accordingly. (See Figure 3.5 for a schematic visual analogue scale for evaluating and scoring histopathological parameters and figures 3.6 to 3.16 for photographic examples (photographs prepared by Dr Abed Zaitoun)). Other parameters such as presence of lymphoid follicles, loss of mucus layer, follicular hyperplasia and whether *H. pylori* colonisation was predominantly on the surface of epithelial cells or in the lumen were also noted. Scoring was blinded to *vacA* mRNA and strain typing results and was carried out simultaneously by me, Sapna Patel and Dr Abed Zaitoun (division of histopathology, University Hospital, Nottingham). Two sections from 2 corporal biopsies were examined and a score was given when all parties were in agreement.

Any correlations between the histopathological findings and *vacA* transcript level were assessed using the real-time PCR data shown in Table 3.1. The *vacA* transcript level in the gastric biopsy was normalised to the *16S* rRNA level in the same biopsy to give *vacA* mRNA level per bacterial cell.

To control for the difference in toxicity between strain types, the analysis was limited to i1-type strains only, as the i1-type has been shown to be the best independent marker of toxicity (Rhead *et al.*, 2007).

Data were analysed with Minitab 14 software. The Mann-Whitney U test was used to assess the association between transcript levels and histopathological parameters and disease. Differences were considered statistically significant for *P* values less than or equal to 0.05.

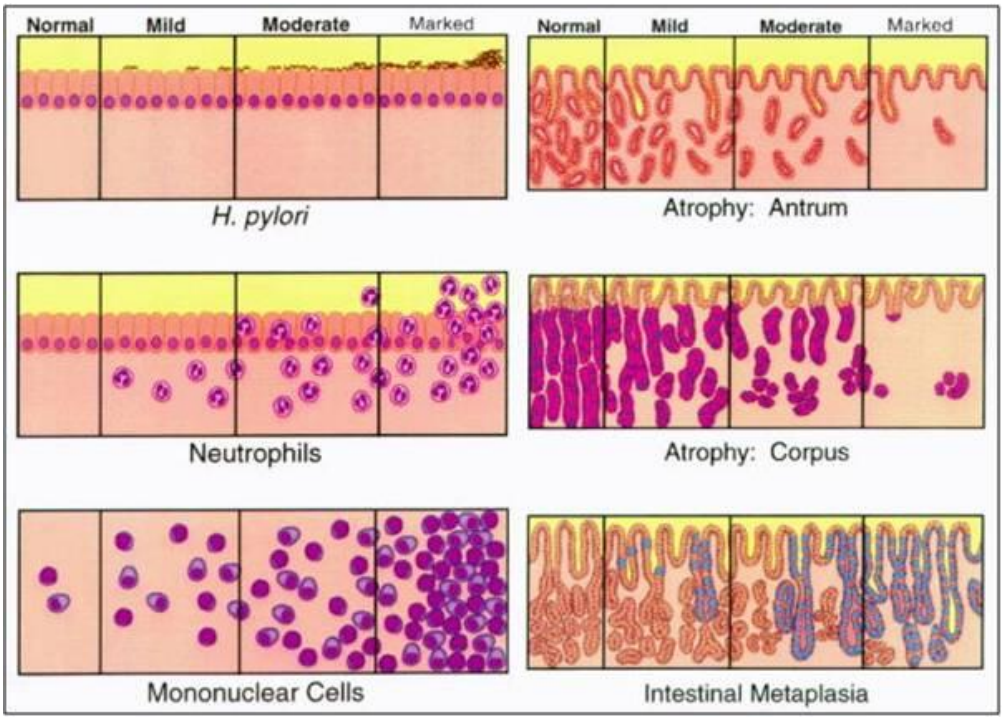


Figure 3.5 Sydney scoring system, using the visual analogue scales: The observer should attempt to evaluate one feature at the time. The most prevalent appearance on each side should be matched with the graded panel that resembles it most closely. Observers should keep in mind that these drawings are not intended to represent realistically the histopathological appearance of the gastric mucosa; rather, they provide a schematic representation of the magnitude of each feature and, as such, have certain limitations. Thus, for example, the decreasing thickness of the mucosa usually observed with increasing atrophy is not depicted realistically. Particularly with *Helicobacter pylori* and neutrophils, there may be a considerable variation of intensity within the same biopsy sample; in such cases, the observer should attempt to average the different areas and score the specimen accordingly (taken from (Dixon *et al*., 1996)).

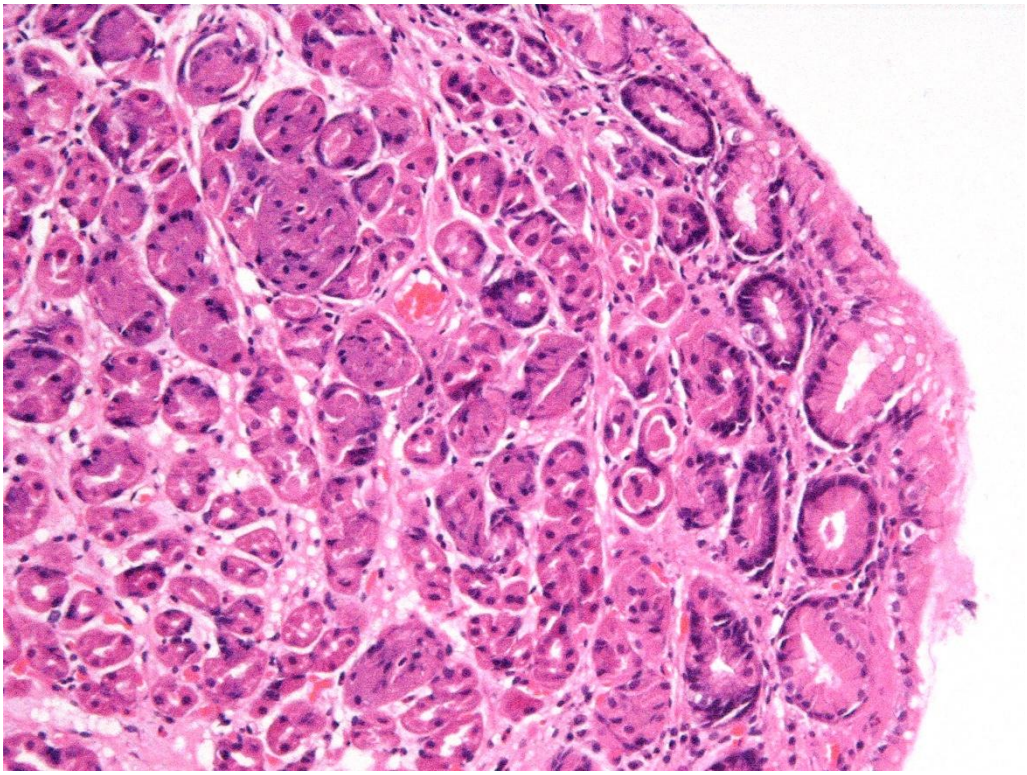


Figure 3.6 Slide showing normal gastric mucosa, not colonized by *H. pylori*. Grade 0 inflammation, activity, atrophy, intestinal metaplasia and *H. pylori* density. Slide stained with haematoxylin and eosine stain (H&E); x 10 magnification.

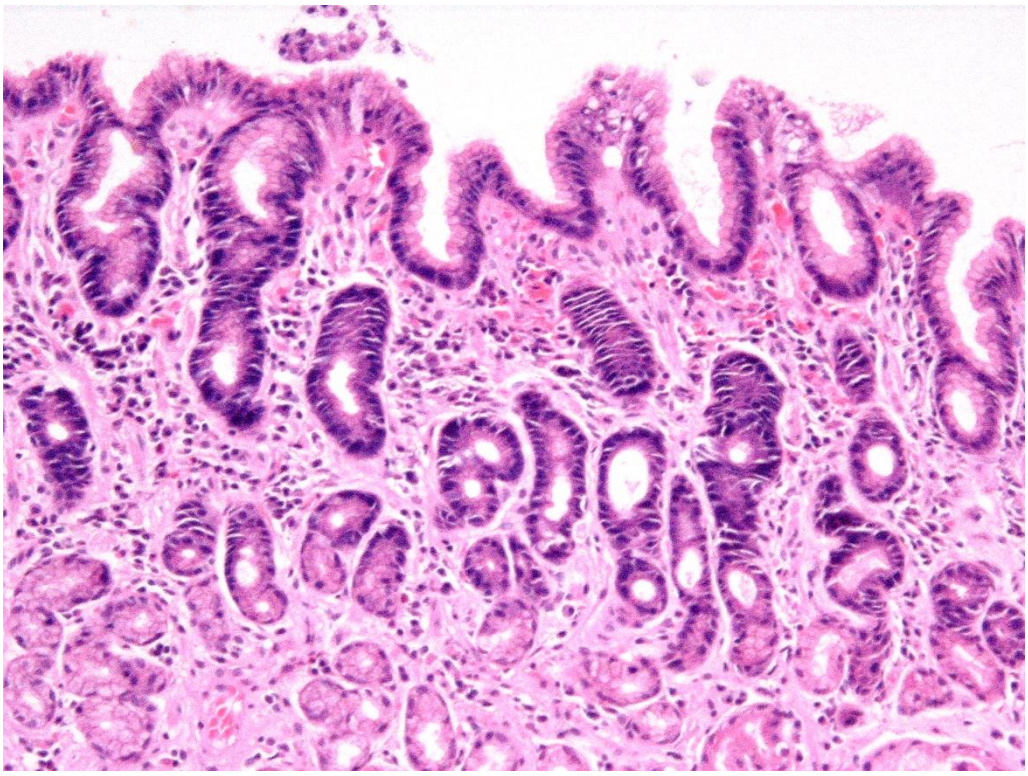


Figure 3.7 Slide showing active chronic *H. pylori* gastritis. The gastric mucosa contains low numbers of lymphocytes and plasma cells (grade 1 inflammation), no signs of polymorphonuclear activity (grade 0 activity), atrophy (grade 0 atrophy) or intestinal metaplasia (grade 0 intestinal metaplasia). Slide stained with H&E; magnification x 20.

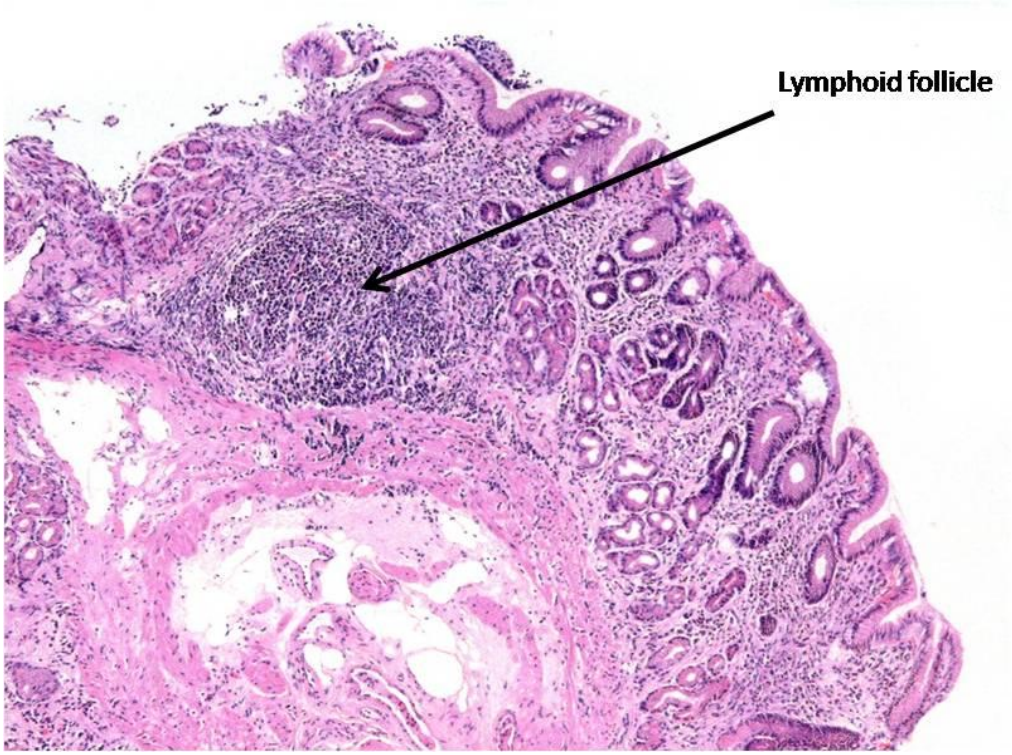


Figure 3.8 Slide showing moderate chronic antral gastritis with lymphoid follicle. The low power view shows moderate chronic inflammation (grade 2) and glandular atrophy (grade 2). Slide stained with H&E; magnification x10.

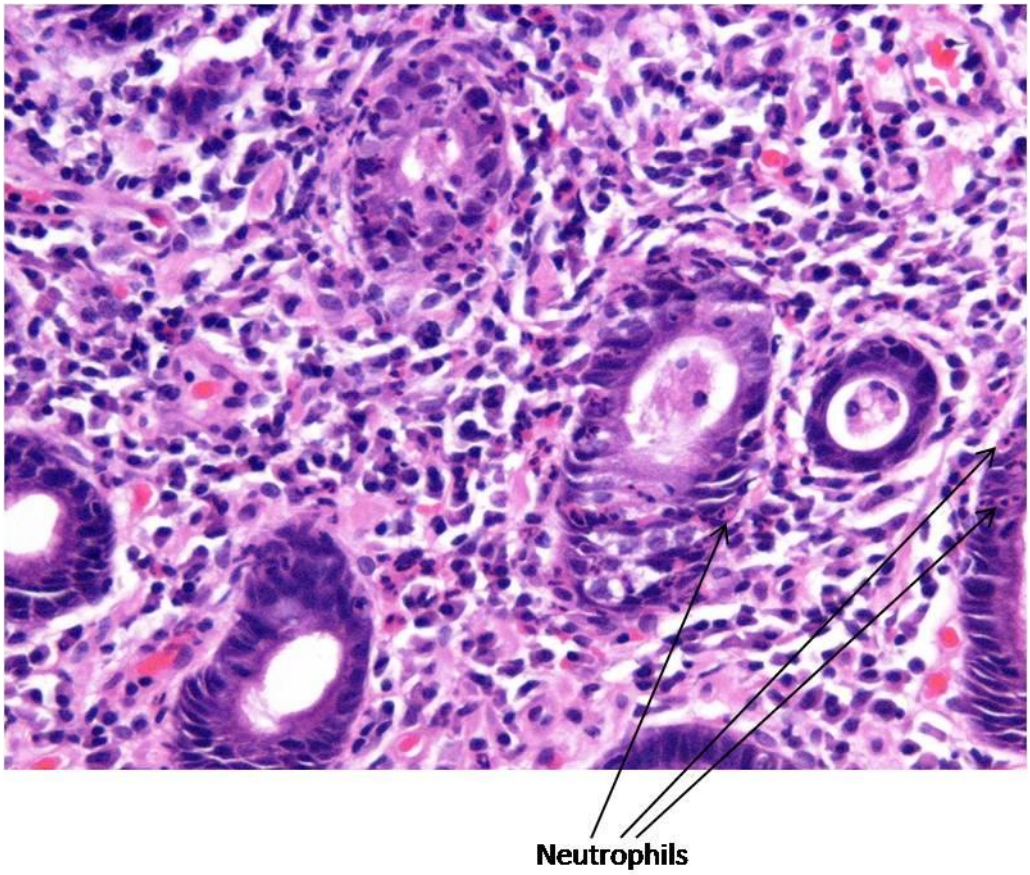


Figure 3.9 Slide showing activity in chronic *H. pylori* antral gastritis. Neutrophils are marked with an arrow. Slide stained with H&E; magnification, x 20.

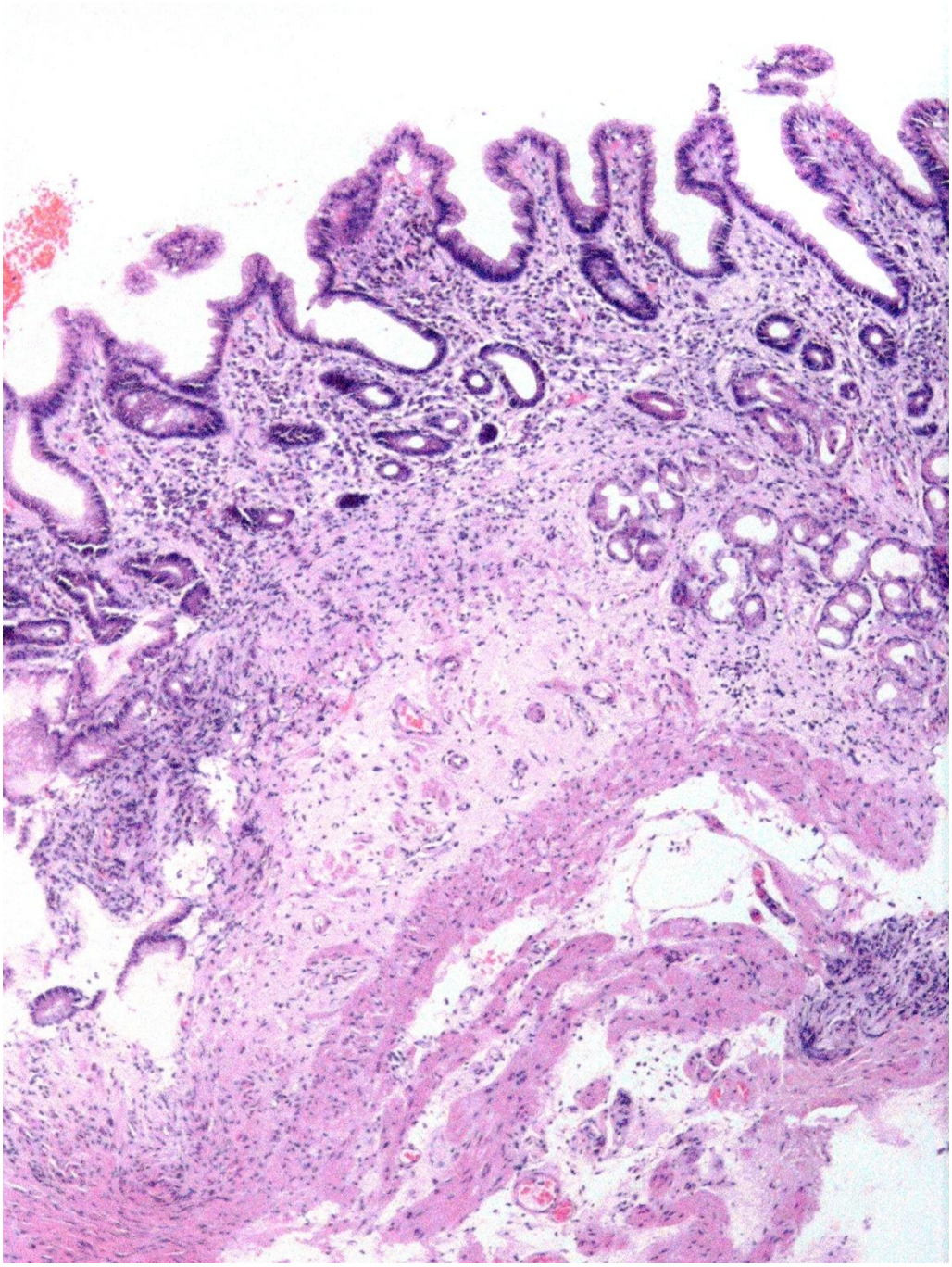


Figure 3.10 Slide showing mild chronic antral *H. pylori* gastritis. The low power view shows mild inflammation (grade 1) with low grade atrophy (grade1). Slide stained with H&E; magnification x 10.

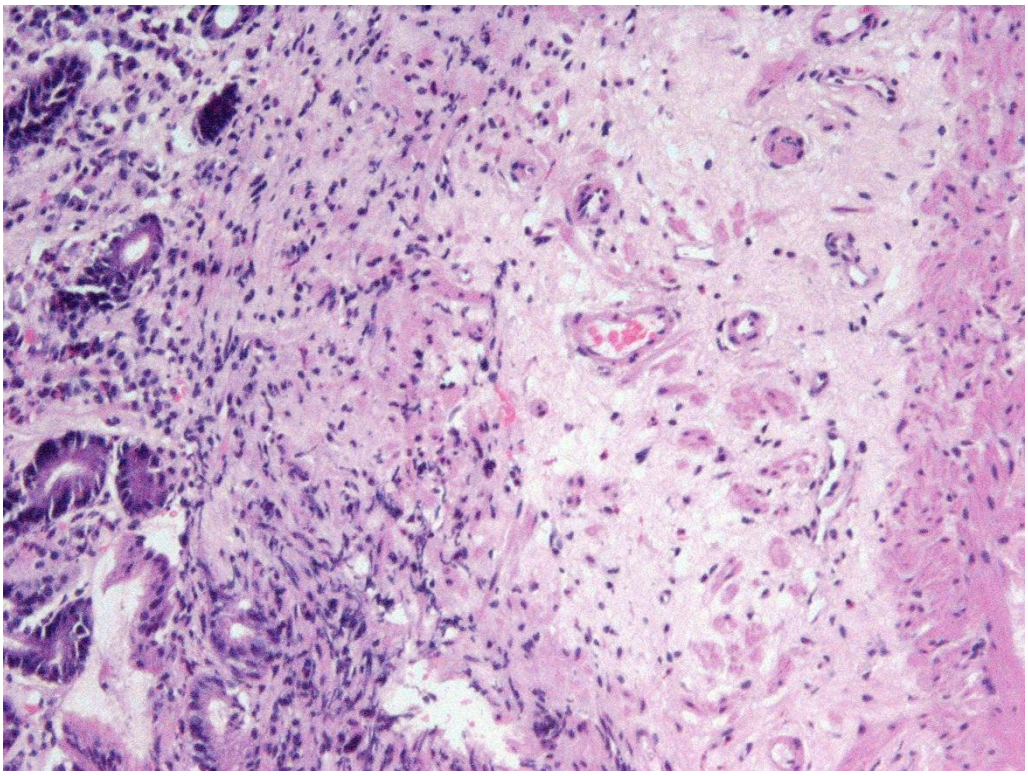


Figure 3.11 Slide showing antral gastritis with marked atrophy (grade 3). Slide stained with H&E; magnification, x 20.

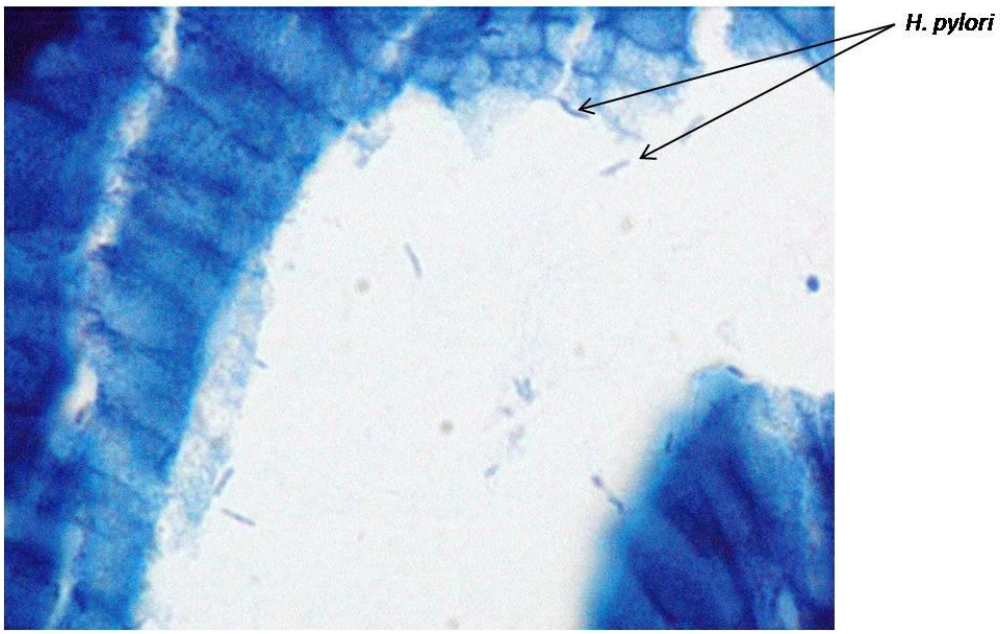


Figure 3.12 Slide showing grade 2 *H. pylori* colonization density. *H. pylori* marked with an arrow. Slide stained with toludine blue; magnification, x 100.

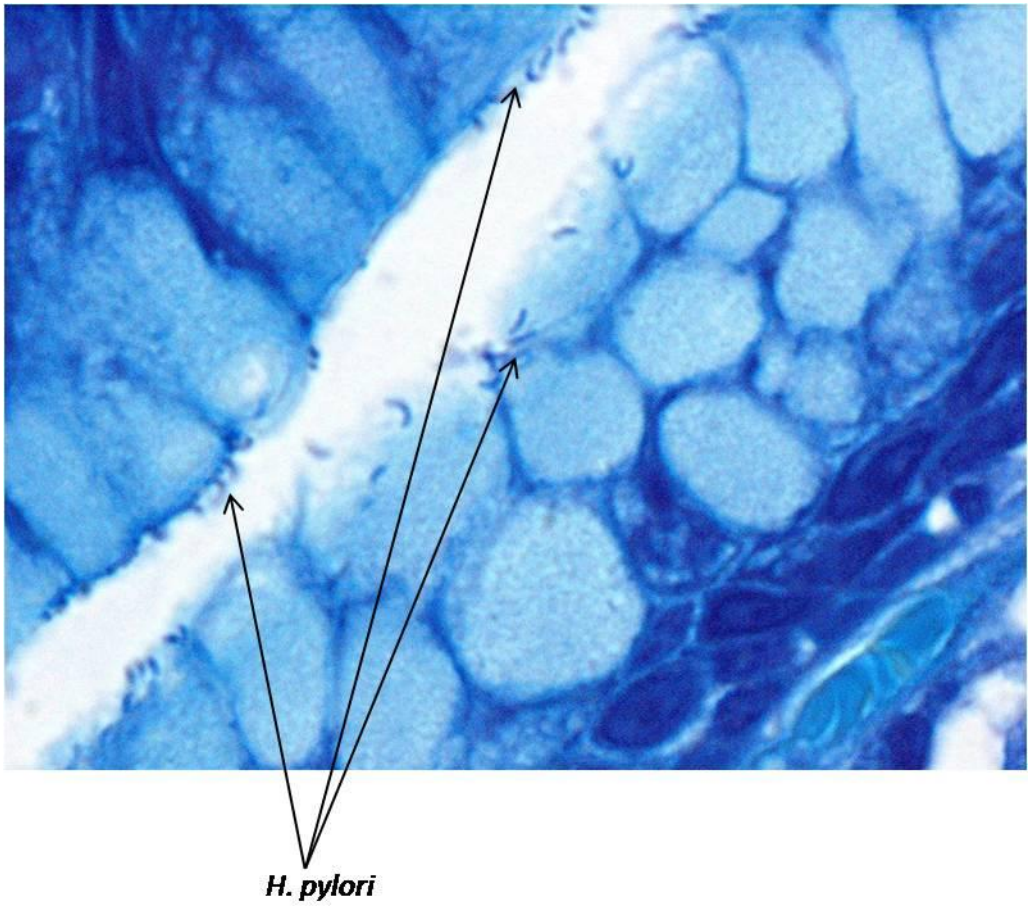


Figure 3.13 Slide showing grade 2 *H. pylori* colonization density. *H. pylori* marked with an arrow. Slide stained with toludine blue; magnification, x 100.

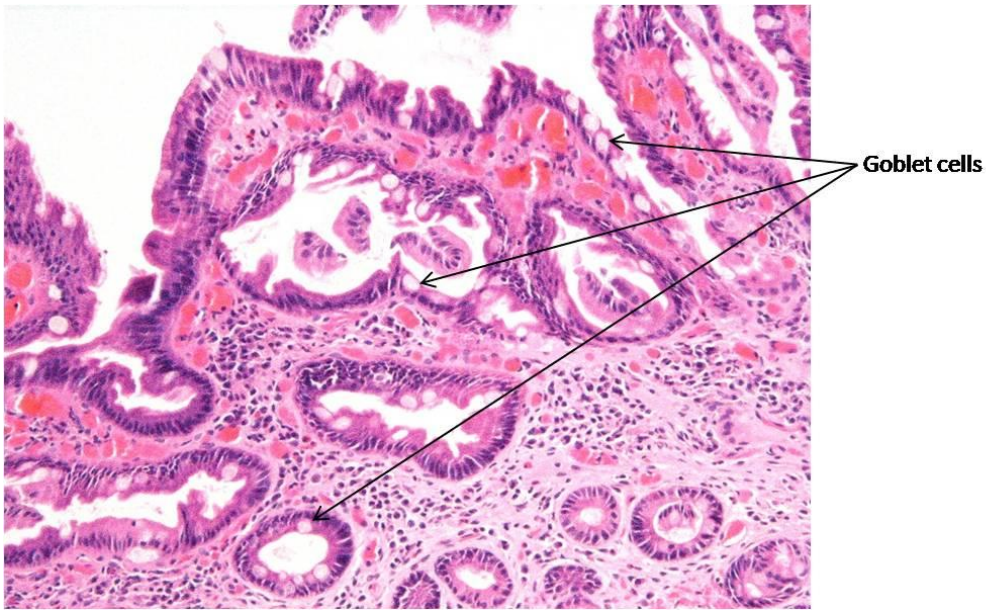


Figure 3.14 Slide showing moderate chronic inflammation and atrophy (grade 2) and marked intestinal metaplasia (grade 3). Goblet cells (an indicator of the presence of intestinal metaplasia) are marked with arrows. Slide stained with (H&E); magnification, x 10.

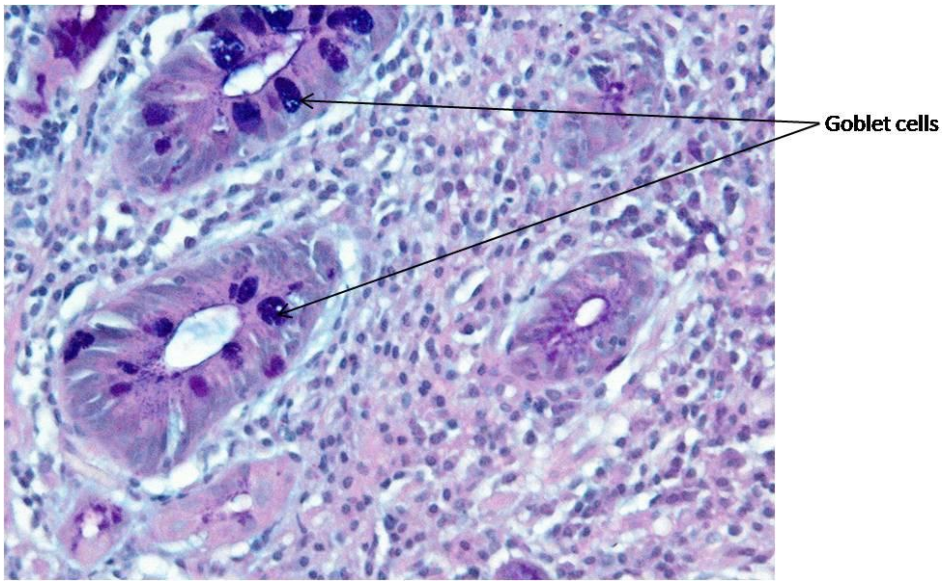


Figure 3.15 Slide showing antral gastritis with mild intestinal metaplasia (grade 1). Goblet cells are marked with arrows. Slide stained with alcian blue/PAS; maginification, x 40.

Figure 3.16 Slide showing antral gastritis with marked intestinal metaplasia (grade 3). Goblet cells are marked with arrows. Slide stained with alcian blue/PAS; maginification, x 40.

3.2.4 *vacA* transcript level is associated with the severity of inflammation, neutrophilic activity and atrophy and the density of *H. pylori* infection

Among the 25 patients infected with an i1-type strain tested, 12 had mild inflammation, 8 had moderate inflammation and 5 had severe inflammation. There were no patients with grade 0 inflammation in the corpus. Comparing the degree of inflammation and the level of *vacA* transcription in the gastric biopsies, it was observed that *vacA* transcript levels were significantly higher in patients with moderate to severe inflammation (median relative *vacA* 5.11) titre compared to those with mild inflammation (median relative *vacA* titre 0.00 (P = 0.006)). *vacA* mRNA levels were significantly lower in patients with mild inflammation compared to those with moderate or severe inflammation (P = 0.013 and P = 0.008, respectively). The median levels of *vacA* were 3.74 and 7.92 in patients with grade 2 and grade 3 inflammation, respectively. However, this difference was not statistically significant.

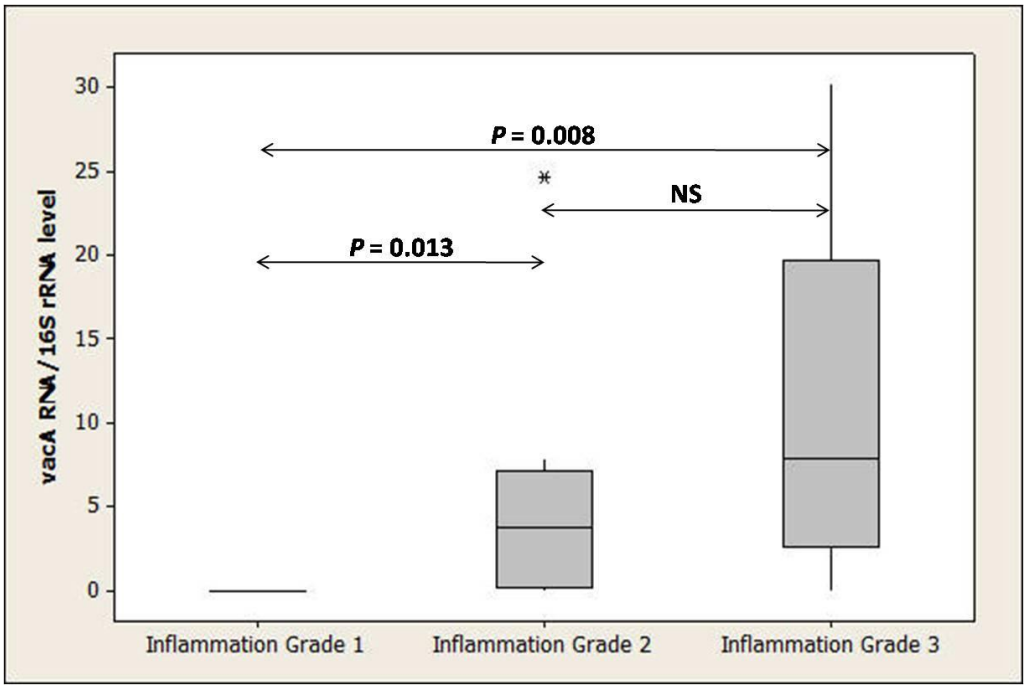
Seventeen out of twenty five patients infected with i1-type strains had neuotrophilic activity in the corpus. Six of those had mild activity, and eleven showed moderate activity. No patients within the group had severe activity. Similarly to findings for inflammation, *vacA* transcript levels were significantly higher in patients with polymorphonuclear activity compared to those with no activity (P = 0.0016). *vacA* levels were significantly lower in patients with no polymorphonuclear activity compared to those with grade 1 or grade 2 activity

(P = 0.05 and P = 0.0007, respectively). *vacA* transcript levels were not significantly different between individuals with mild versus moderate activity.

Six out of the twenty five patients tested who were infected with an i1-type strain had grade 1 atrophy. The other 19 patients showed no signs of atrophy in the corpus. When the relationship between the level of *vacA* transcript and the presence of atrophy in the gastric biopsies was assessed, it was observed that *vacA* transcript levels were significantly higher in patients with atrophy than those with no atrophy P = 0.017 (median values 6.49 and 0.0 respectively).

Fifteen out of twenty five patients infected with i1-type strains had grade 1 infection density in the corpus. One had grade 2 infection density and nine had grade three infection density. As there was only one patient in the grade 2 density group, patients with grade 2 and 3 infection density were combined. *vacA* transcript levels were significantly higher in patients with an infection density of grade 2 and 3 compared to those with a grade 1 infection density (P = 0.0056) (median values 5.16 and 0.0 respectively).

A



B

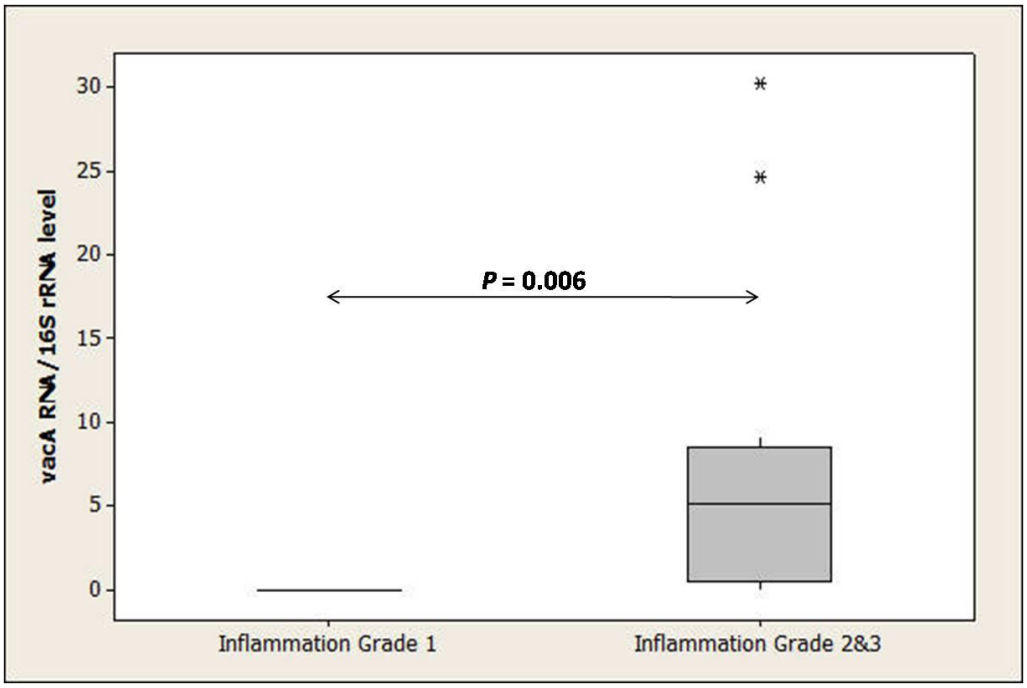
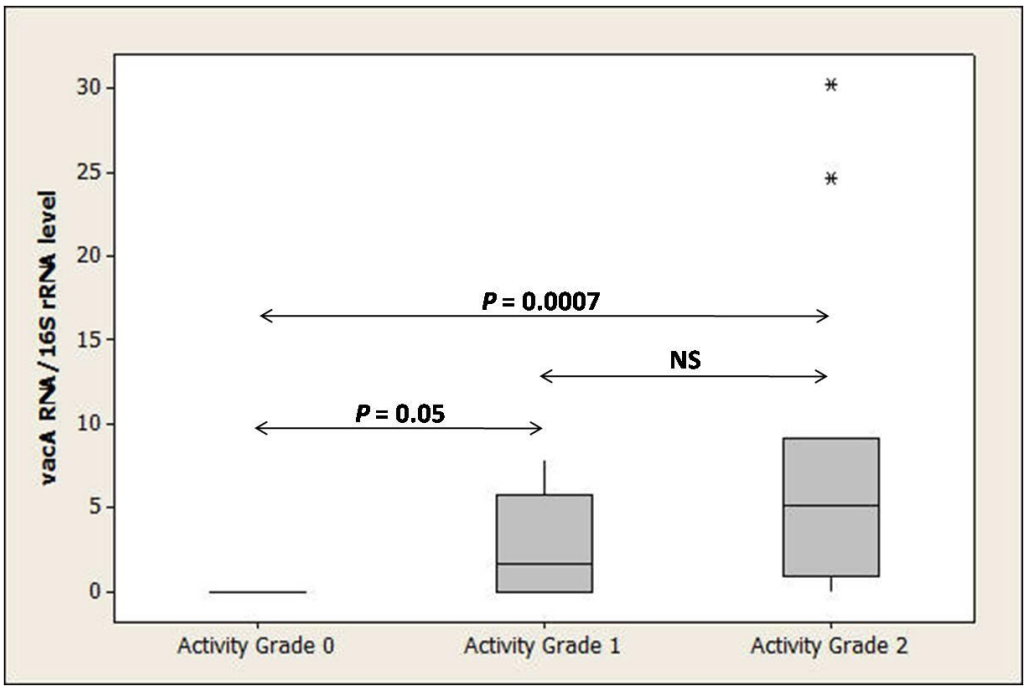


Figure 3.17 Box and whisker plot showing the *vacA* mRNA level of strains in gastric biopsies taken from patients with mild, moderate and severe inflammation (A) and mild versus moderate and severe inflammation (B). P values were assessed by the Mann-Whitney U test. Data are expressed as median with interquatile ranges (box 25-75%; bars 10-90%).

A



B

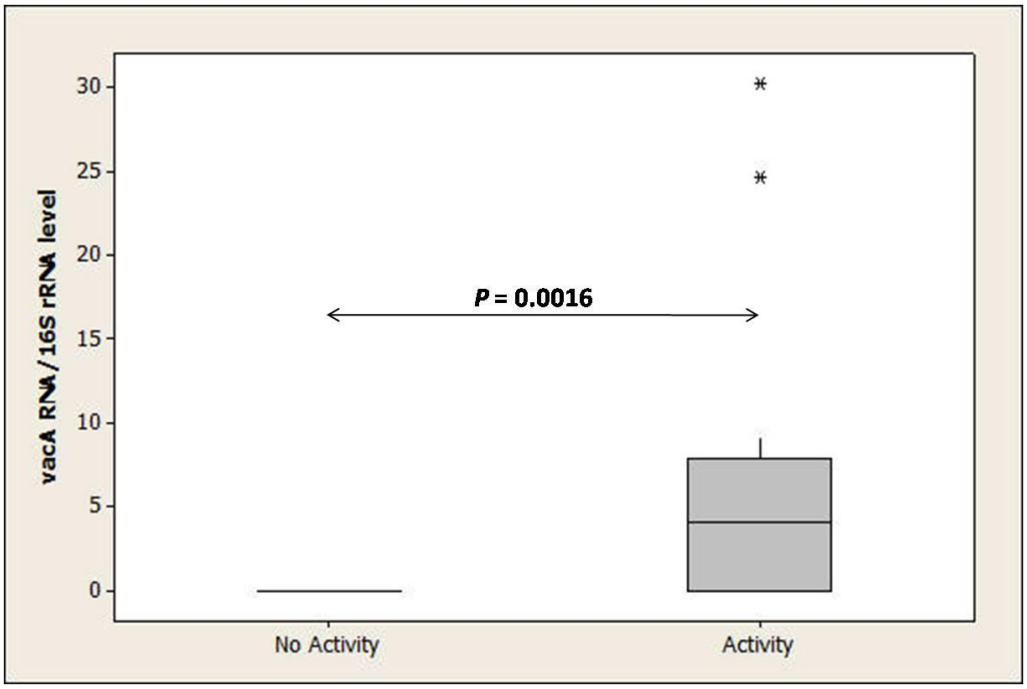


Figure 3.18 Box and whisker plot showing the *vacA* mRNA level of strains in gastric biopsies taken from patients with no, mild and moderate activity (A) and activity versus no activity (B). P values were assessed by the Mann-Whitney U test. Data are expressed as median with interquatile ranges (box 25-75%; bars 10-90%).

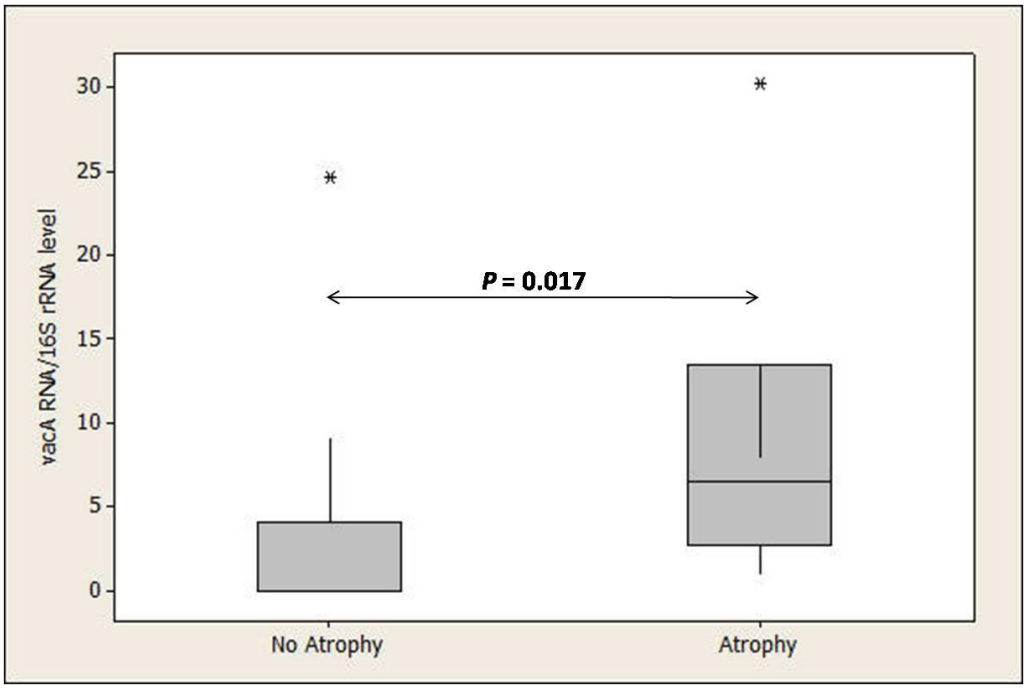


Figure 3.19 Box and whisker plot showing the *vacA* mRNA level of strains in gastric biopsies taken from patients with atrophy and no atrophy. P values were assessed by the Mann-Whitney U test. Data are expressed as median with interquatile ranges (box 25-75%; bars 10-90%).

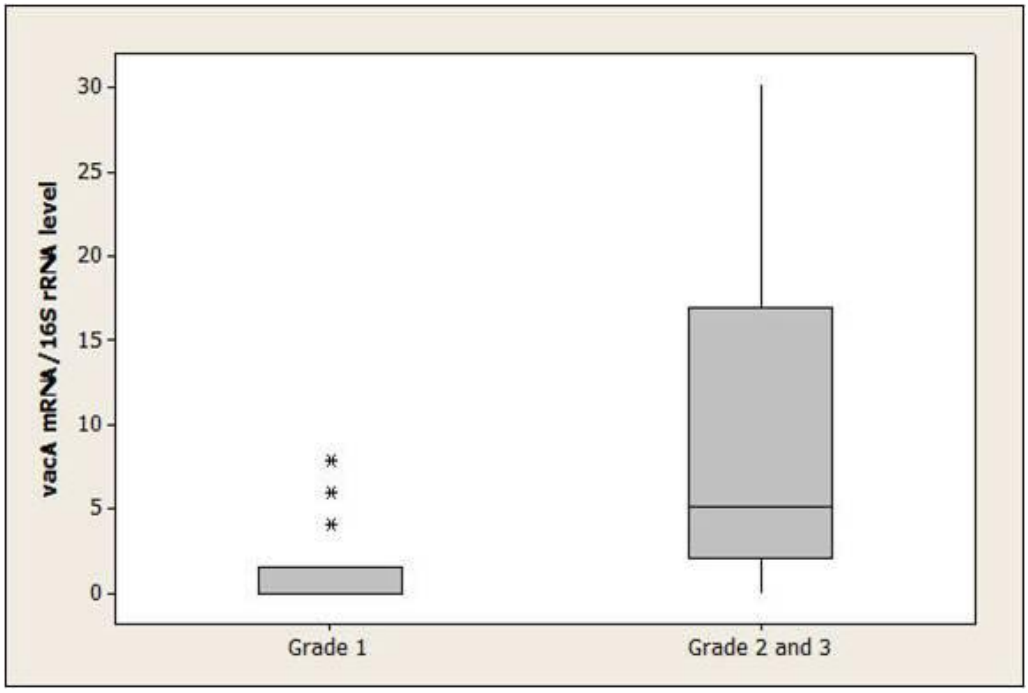


Figure 3.20 Box and whisker plot showing the *vacA* mRNA level of strains in gastric biopsies taken from patients with grade 1 infection density and grade 2 or 3 infection density. P values were assessed by the Mann-Whitney U test. Data are expressed as median with interquatile ranges (box 25-75%; bars 10-90%).

3.2.5 Association between *vacA* transcript levels and other histopathological parameters

Only two patients in the study had intestinal metaplasia, so the numbers were not sufficient for statistical analysis. However, the two patients were infected with s1/i1/m1 type strains and the median relative *vacA* level in the group with intestinal metaplasia was 6.51 compared with a median value of 0.0 in the group of patients with no intestinal metaplasia.

Using histopathology to assess the primary location of the *H. pylori* colonisation in the corpus, it was observed that the strains mainly colonised the surface of the epithelial cells in 11 out of 21 patients, the lumen in 7 patients; and in the other 3 patients the location of colonisation was mixed. There were no significant differences between *vacA* transcript levels of strains that predominantly colonised the epithelial cell surface compared to those that colonised the lumen.

3.2.6 Association with disease

During endoscopy, disease severity and other observations were noted alongside the patients' symptoms. Patients who showed no pathology were classified as normal and those with ulcers or erosions were categorised into the erosive disease group. Samples were not taken from patients who had taken antibiotics or proton pump inhibitors two weeks prior to the endoscopy. Sixteen out of twenty six patients infected with i1-type strains showed signs of disease, the other 10 patients showed no pathology.

When the relationship between the level of *vacA* transcript and the presence of erosive disease was assessed, it was observed that the median level of *vacA* transcript was 1.23 in the disease group and 0.00 in the normal group. However, the differences between the two medians were not found to be statistically significantly different (P = 0.5800).

A

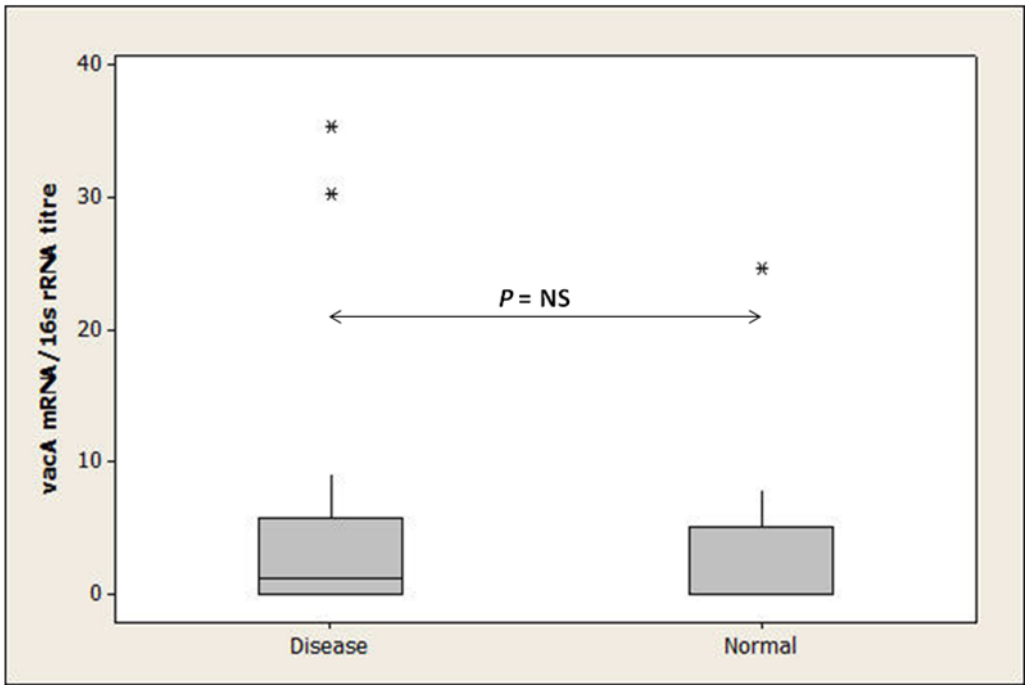


Figure 3.21 Box and whisker plot showing the *vacA* mRNA level in gastric biopsies taken from patients with disease and no disease. P values were assessed by the Mann-Whitney U test. Data are expressed as median with interquartile ranges (box 25-75%; bars 10-90%).

3.3 Discussion

Infection with *H. pylori* results in chronic gastritis and can lead to diseases such as peptic ulcer disease, gastric cancer and gastric MALT lymphoma (Dunn *et al*., 1997, Spencer *et al*., 1997, Graham *et al*., 2000). Many studies have investigated the association with *vacA* type and disease outcome (Atherton *et al*., 1997). In Western populations, s1/i1/m1 type strains are associated with higher levels of inflammation, peptic ulcer disease and gastric cancer (Rhead *et al*., 2007). The association between *vacA* type and disease in East Asian and Southeast Asian populations is not as clear as strains are predominantly of the *vacA* s1/i1 type in these populations. However, it is thought that the high prevalence of these more virulent strain types in these populations may contribute to the high incidence of gastric cancer within this region. Although *vacA* type has been shown to be a good marker of toxicity, not all individuals infected with more virulent strains go on to develop disease. Heterogeneity in *vacA* levels is known to exist and is thought be a factor that contributes to toxicity; large amounts of purified VacA induce ulcer-like erosions in mice, and VacA increases gastric ulcer formation in experimentally infected gerbils (Marchetti *et al*., 1995, Ogura *et al*., 2000). This chapter investigates the heterogeneity in *vacA* mRNA levels between different strains *in vivo* and assesses the associations between *vacA* transcript level and the disease severity of the infected individual determined by histopathology.

In this study a significant positive association was seen between the level of *vacA* transcript produced per *H. pylori* cell and the severity of inflammation,

activity and the presence of atrophy in the corpus of infected patients. VacA has been shown directly damage the gastric epithelium, a gastric mucosal inflammatory response may occur as a consequence of the VacA mediated damage. Additionally, the findings of several studies provide evidence that VacA may be directly involved in proinflammatory signalling. VacA induces p38 activation in gastric epithelial cells, T-cells, macrophages and neutrophils (Boncristiano *et al*., 2003). p38 activation has been shown to increase production of COX-2 which can lead to the generation of proinflammatory prostanoids (Hisatsune *et al*., 2007, Boncristiano *et al*., 2003). VacA has also been found to stimulate proinflammatory cytokines and chemokines production by mast cells and eosinophils contributing to inflammation (Supajatura *et al*., 2002, Kim *et al*., 2007). Previous studies in animal models have found that gastric pathology resembles that of human gastric disease in infections with toxigenic strains but not with non toxigenic strains (Marchetti *et al*., 1995). In a mouse model of infection oral administration of *H. pylori* sonicate from toxigenic strains reproduced several aspects of the histological lesions observed in biopsies from patients with gastric ulcer disease. These included epithelial vacuolation, infiltration of mononuclear cells into the lamina propria, loss of gastric gland structure and ulceration (Telford *et al*., 1994b, Ghiara *et al*., 1995). Sonicate of a non-toxigenic strain did not induce histological lesions. Indicating that the cytotoxin is necessary to induce gastric damage (Telford *et al*., 1994b). Oral administration of purified VacA into the same mouse model induced the same histological lesions as those obtained with the crude extract, although inflammatory cell infiltration was not observed. A study by Supajatura *et al* (2002) confirmed the findings that purified VacA

induces histological lesions in the murine gastric mucosa, however no neutrophil infiltration was observed in this particular study (Supajatura *et al*., 2002). Interestingly, significantly fewer gerbils suffered from gastric ulcers when infected with an isogenic *vacA* null mutant when compared to those infected with an isogenic wild type toxigenic strain (Ogura *et al*., 2000). However the severity of gastritis in the gerbils infected with a null mutant was not significantly different from that seen in gerbils infected with the wild type strain (Ogura *et al*., 2000). Superficially, this appears difficult to reconcile with my results, but the level of *vacA* transcription and production by the strains used in the study is not stated. The strain used may have been a low transcriber of *vacA* so deletion of the *vacA* gene may not have had a significant effect on the toxicity of the strain *in vivo* and could explain the inconsistency between the other studies on the importance of VacA in gastric disease in animal models. A difference in the inflammatory response between species may also be responsible.

The differences in inflammation and activity observed between this study and previous animal studies on VacA may partly be explained by the fact that many studies in animal models routinely infect animals with high bacterial loads. Also histopathological changes were observed within hours of infection or exposure to the toxin (Ogura *et al*., 2000, Ghiara *et al*., 1995, Salama *et al*., 2001, Supajatura *et al*., 2002). Furthermore, delivery of VacA to the gastric mucosa in an animal model of acute mucosal injury which consists of large pulse administrations of bacterial sonicates or purified cytotoxin is likely to be very different from the targeted slow release occurring during *H. pylori*

infection. Moreover, *H. pylori* infection in humans is a slow progression, gastric disease is manifested after prolonged infection, and the early inflammatory changes are difficult to recognise (Supajatura *et al*., 2002). Murine T-cells are resistant to the effects of VacA (Algood *et al*., 2007) indicating that there may be differences in the immune response to *H. pylori* infection between species.

A positive correlation between the *vacA* level of an infecting strain and the density of infection in the corpus was observed. Several lines of evidence indicate that VacA may enable *H. pylori* colonization of the gastric mucosa. Competition experiments with an isogenic *vacA* null mutant and a wild type strain in a mouse model indicated that VacA may play a role in colonisation of the gastric mucosa (Salama *et al*., 2001). Salama *et al*. (2001) suggested that VacA causes tissue damage leading to alteration of the local pH and nutrient release allowing *H. pylori* to survive in the mucosa. Another suggestion was that cell damage caused by VacA plays a role in the local inhibition of acid secretion, which facilitates *H. pylori* colonisation (Salama *et al*., 2001). This is supported by a study by Boonjakuakul *et al*., (2005) during *H. pylori* infection of rhesus macaques *vacA* mRNA levels were higher one week after infection than 4-6 weeks after infection (Boonjakuakul *et al*., 2005). *vacA* was also found to be one of the most highly expressed putative virulence factors in *H. pylori* positive human gastric biopsy specimens out of a total of 18 virulence factors assessed (Boonjakuakul *et al*., 2004).

Several functions have been ascribed to VacA which include modulating the trans-epithelial resistance of cell-cell junctions to release essential nutrients from the epithelium (Papini *et al*., 1998). VacA has also been shown to form pores in the cell membrane, allowing egress of anions and urea which enables *H. pylori* to protect itself from the acidity of the gastric environment (Iwamoto *et al*., 1999, Szabo *et al*., 1999, Tombola *et al*., 1999, Tombola *et al*., 2001). Several studies have suggested that VacA may play an important role in modulating the immune response during infection by inhibiting the activation and proliferation of T lymphocytes (Akada *et al*., 2000, Fan *et al*., 1994) affecting B lymphocyte antigen presentation (Molinari *et al*., 1998b) and modulation of the T cell mediated cytokine response (Gebert *et al*., 2003). Collectively these findings indicate that VacA can induce physiological changes that may contribute to pathogenesis and the persistence of *H. pylori* in the gastric niche. However, the exact role of the cytotoxin in gastric pathogenesis *in vivo* remains to be determined.

Only two patients in the study had intestinal metaplasia, consequently there were insufficient numbers for statistical analysis. However, the two individuals with intestinal metaplasia were both infected with strains of the toxigenic *vacA* type s1/i1/m1 and had relative *vacA* levels within the top 8 highest transcribers of *vacA*.

No association was found between *vacA* transcript level and disease outcome as determined during the endoscopy. Observations of disease during endoscopy only show whether a patient has observable pathology on the day of the

endoscopy, it may be the case that the patient may present with disease at another point in time. Investigating histopathological changes enables quantification of different parameters of disease progression at earlier time points and although a patient does not show signs of ulcer disease at the present time, it may be that in time disease will develop. This may explain the seeming disparity between the histopathological observations and disease determined by endoscopy. Furthermore, it could be argued that the investigations of histopathological changes are a more accurate measure of disease progression. Other studies in mice and gerbils showed a positive association between VacA and ulcers, however it is likely that there are differences in the inflammatory response between humans and animal models. In the studies of acute mucosal injury using animal models, large bacterial loads, pulse administrations of bacterial sonicates or purified cytotoxin are used which are not representative of virulence factor release in the human stomach. Another explanation is a type two error, the study may not be big enough to show a statistically significant association.

The *vacA* genotype of an infecting strain has been found to be a good marker of disease. This chapter showed that there is a relationship between *vacA* transcript levels produced by a strain and the severity of inflammation, activity and atrophy in the corpus. Several putative virulence factors including the *cag* pathogenicity island, BabA and in-frame OipA alleles are present in strains of the more toxic s1 *vacA* type more frequently than in strains of the non-toxic *vacA* s2 type (Atherton *et al*., 1995, van Doorn *et al*., 1998, Gerhard *et al*., 1999, Cao and Cover, 2002, Yamaoaka *et al*., 2002, (Dossumbekova *et al*.,

2006). It is therefore difficult to determine which of these putative virulence factors is the most important in the pathogenesis of gastroduodenal disease. However other factors such as variations in acid production, the immune response of the infected individual, environmental factors such as smoking and diet and other *H. pylori* virulence factors may also have an effect on histopathological severity and the disease status of the patient. It is likely that the development of disease in humans is not due to the action of one single factor and that a combination of these factors contribute to development of disease. More studies are required to assess the relative importance of these other factors in *H. pylori* associated disease.

In conclusion this chapter has shown that the level of *vacA* transcript produced by a strain is positively associated with the severity of inflammation, atrophy and neutrophil activity, and with the density of infection in the corpus. Differences in *vacA* expression between *H. pylori* strains *in vivo* could explain the imperfect correlation between *vacA* type and disease. Host factors and other *H. pylori* virulence factors may also have an effect on histopathological severity and the disease status of the patient. However, more studies are required to assess the importance of these other factors in *H. pylori* associated disease.

3.3.1 Limitations

3.3.1.1 Sample size

This study assessed the *vacA* mRNA level present in corporal biopsies taken from 39 patients, only 25 of those were of the *vacA* i1 type. The analysis could

have been improved if more samples had been available for testing. Unfortunately, the number of gastric biopsies that could be taken for research was limited by ethical constraints. In some cases in this chapter, correlations that were not statistically significant showed trends which may reach significance, if additional data points were included. Gastric biopsies were sampled from the same sites in all patients involved in the study to control for differences in colonisation density between different areas of the corpus. It would be interesting to analyse samples taken from different sites in the corpus to determine whether *vacA* mRNA levels are similar in different sites within the corpus. Unfortunately, the limited sample size meant that this was not possible.

This chapter investigated *vacA* mRNA levels using quantitative real-time PCR, ideally transcript levels would be backed up by another method such as Northern blotting. Similarly, it would be interesting to determine whether *vacA* transcript levels correlate with VacA protein levels by Western blotting or ELISA. However, the amount of gastric tissue available in this study meant that this was not possible.

Patients regularly taking NSAIDs were excluded from this study. However, we could not control for intermittent use or for cases where patients had taken NSAIDs unwittingly (e.g. in cold remedies). NSAIDs are known to cause peptic ulcers. Thus, we cannot rule out the possibility that some disease conditions identified in this study were due to unrecorded NSAID use. The age and sex of the patient along with the *cagA* status of the strain were taken into

account, however other factors such as diet, host genetics and smoking all have an effect on severity of gastric disease, unfortunately this data was not available for these patients and so could not be controlled for in this study.

3.3.1.2 Real-time PCR

Reverse transcription followed by quantitative real-time PCR is the most sensitive and reliable method for quantifying low abundant transcripts in tissues (Freeman *et al*., 1999, Steuerwald *et al*., 1999). Several methods of analysis are available to quantify the mRNA content in a sample. The level of gene expression can be measured using an absolute or a relative quantification. Absolute quantification relates the level of fluorescence to an input copy number using a calibration curve which can be derived from dilutions of spiked tissue samples, recombinant DNA or RNA or PCR products (Bustin, 2000, Fronhoffs *et al*., 2002). In this chapter, differences in *vacA* mRNA levels between patients were assessed using relative quantitation which determines differences in mRNA levels across multiple samples and expresses it relative to that of a control sample which is included on every run. The Pfaffl method of analysis was used which also takes into account the amplification efficiency of a gene in the particular sample type. Target gene expression is adjusted to the expression of a house-keeping gene that is constitutively expressed. In this study *16S* rRNA was used and is commonly used as the housekeeping gene of choice in gene expression studies in bacteria.

The level of expression of a gene is based on the distinct cycle that the fluorescence of a sample crosses a threshold level of fluorescence which is selected by the user at a level where amplification is exponential. For accuracy, the threshold needs to be set as low as possible and needs to be the same on each real-time run. In this study the threshold was set at 0.01.

vacA transcript was not detectable in 18 of the biopsies tested. The limit of detection of *vacA* was found to be 0.18. This is 8.2 fold lower than that of sample 223 which had the lowest detectable level of *vacA* in this study. Samples that had no detectable *vacA* transcript were given a value of zero. The Mann Whitney U test which ranks the samples in order or transcript level was used to compare mRNA levels between disease groups so that designating a value of zero to samples with no detectable *vacA* transcript would not affect the analysis. *16S* rRNA was detectable in all of the biopsies tested indicating that *H. pylori* was present in the biopsy and that the RNA had not degraded.

3.3.2 Future work

In Western populations such as the USA and Western Europe, strains of the *vacA* s1/m1 type have been shown to be associated with peptic ulceration, and gastric cancer (Atherton *et al*., 1995, Atherton *et al*., 1997, Rhead *et al*., 2007, Basso *et al*., 2008). The *vacA* i1 type is also a risk factor for peptic ulcer disease, duodenal ulcer disease and gastric cancer (Basso *et al*., 2008, Rhead *et al*., 2007). In East Asian and Southeast Asian countries the association between *vacA* allelic diversity and clinical outcome is not well established as the

population is predominantly infected with strains of the s1/i1 type (Maeda *et al*., 1998, Yamaoka *et al*., 1998). There is a high incidence of gastric cancer in this population which could be explained by the high prevalence of the more virulent *vacA* s1/i1 type strains (Rhead *et al*., 2007). However, not all individuals infected with more virulent strains go on to develop disease. It would be interesting to repeat the study carried out in this chapter on different populations to determine whether differences in *vacA* expression between *H. pylori* strains of the same *vacA* type *in vivo* could explain the imperfect correlation between *vacA* type and disease.

Studies have shown that the pattern of gastritis is important in determining disease outcome with antral predominant gastritis being associated with duodenal ulceration and a pangastritis being epidemiologically linked with gastric ulcers and gastric adenocarcinoma. It would be interesting to look at whether there is a correlation between the level of *vacA* transcript in the antrum and in the corpus, and to investigate whether there is a relationship between *vacA* transcript level and the pattern of gastritis.

Chapter 4

4 Characterisation of the effect of natural polymorphisms in the *cysS – vacA* intergenic region on *vacA* transcription levels *in vitro* and *in vivo*.

4.1 Introduction

Heterogeneity in *vacA* transcription between strains is known to exist; Ayala *et al.,* (2004) observed 28 fold differences in *vacA* transcription between 8 different *H. pylori* strains cultured *in vitro* using real-time PCR. Using quantitative primer extension analysis, Forsyth *et al.,* (1998) found that *vacA* is transcribed in both type s1/m1 (Tox+) and s2/m2 (Tox-) strains, but type s1/m1 (Tox+) strains produce significantly higher levels of toxin than s2/m2 (Tox-) strains (Forsyth *et al.,* 1998). This difference in transcript level correlated with differences in VacA protein level (Forsyth *et al.,* 1998).

Prokaryotic gene transcription requires the RNA polymerase holoenzyme with a specific sigma subunit, which is required for making sequence specific contacts with the promoter DNA. Prokaryotic promoters are generally composed of 2 short conserved sequences (termed the -10 and -35 boxes which are located 10 nucleotides and 35 nucleotides upstream of the transcription start site, typically a single purine nucleotide (A or G)). Some promoters lacking a conserved -35 sequence depend on an extended -10 region for transcription initiation. The extended -10 region consists of 2 additional bases upstream of the -10 motif. Variations in both the promoter sequence and promoter element spacing influence the level of transcription. Some bacterial promoters contain sequences in addition to the -35 and -10 motifs that can

further modulate transcription by allowing binding of activators or repressors of transcription.

Previous studies have shown that *vacA* transcription is initiated by a single promoter which is in the 285bp intergenic region located between the upstream housekeeping gene, cysteinyl-tRNA synthase (*cysS*) and the start of *vacA* (Forsyth *et al.,* 1998). Sequence analysis of this region from 12 strains revealed that *vacA* has a single TSP which is located 119 nucleotides upstream of the AUG start codon, a conserved -35 region (TTTATG), a conserved -10 region (TAAAAA) and a TGN motif adjacent to the -10 motif (Davies *et al.,* 2002) similar to the extended -10 boxes found in *E. coli* (Forsyth *et al.,* 1998).

Forsyth *et al.,* (1999) created specific mutations in the *cysS* – *vacA* intergenic region to assess the importance of specific promoter elements on *vacA* transcription using a *xylE* reporter system. Mutations in the -10 region, where the wild type sequence TAAAAG was changed to AGATCT resulted in a 15 fold reduction in *vacA* level. Changing the -35 motif from the wild type sequence TTTATG to AGATCT resulted in a 4 fold reduction in expression. The specific sequence of the spacer region did not appear to be important in determining transcript levels as substituting CCTTA to GATCT from positions -23 to -19 did not affect expression levels. However spacing appeared to be important as introducing a 10bp insertion between -24 and -23 reduced expression. They concluded that -35 and -10 sequences along with promoter element spacing are important for binding.

Substituting G for T at the -14 position resulted in a 2 fold reduction in *vacA* transcript level, and changing the T to a G at the -12 position resulted in a 4 fold reduction. Deletion of 6 nucleotides (TTTATG) from position -37 to -32 in strain 60190 resulted in a 6 fold reduction in expression. Substitutions at the -13 and -15 positions had no effect on *vacA* transcript levels. This lead to the conclusion that positions -12 and -14 may be important for RNA polymerase binding. Forsyth *et al.,* (1999) also found that 15 out of 17 strains all used the same transcriptional start point. They suggested that the conservation of the adenosine residue at the +1 position may be important as Liu *et al.,* (1994) reported that the identity of the +1 site can affect transcriptional efficiency.

Using promoter exchange experiments Forsyth *et al.,* (1999) also found that Tox- strains under control of a Tox+ promoter showed no difference in *vacA* transcription. When reciprocal changes were made they found that a Tox+ strain under the control of a Tox- promoter resulted in a 65% decrease in *vacA* transcription in the reporter strain. However *vacA* transcription was still 10 fold higher than in the control Tox- reporter strain. They concluded that Tox+ and Tox- strains investigated differ in promoter strength but the differences must be due to changes outside the -35 and -10 motifs, as the two hexamers were identical in both strains studied. They suggested that the difference in *vacA* transcriptional levels between the two strains may be due to a trans-acting repressor in the Tox- strain or an activator present in the Tox+ strain which are absent or reduced in the other strain type.

Ayala *et al.,* (2004) also found that the differences in *vacA* expression between 8 strains *in vitro* were unrelated to -10 and -35 sequences. G. L. Narayanan (2005) analysed the *vacA* promoter sequences of a collection of published strains and found, in agreement with Forsyth *et al.,* (1999) that there appears to be an extended – 10 region. It was also observed that in 4 out of 8 clinical strains there was a substitution from a G to an A at position -14 that makes up part of the extended -10 region. In addition the final base within the -35 motif has a frequent substitution from a G to an A residue.

Stem loop structures have been shown to be important in determining transcript stability in prokaryotes. Stem loop structures can be found at both the 5' and the 3' end of the *vacA* transcript (Emory and Belasco, 1990, Emory *et al*., 1992). All 17 of the Tox+ and Tox- strains tested by Forsyth *et al.,* (1999) encoded potential 3' stem loops structures (Atherton 1995 *et al*., and Cover *et al*., 1994) however these appeared to be well conserved and they found no evidence that these 3' stem loops had any effect on differences in *vacA* transcription between strains.

To investigate whether the differences in *vacA* transcription between strains were due to mRNA stability, Forsyth *et al.,* (1999) treated the 2 strains with rifampicin to inhibit RNA polymerase activity and assessed transcript levels using primer extension analysis, although this data was inconclusive. Using a sequence alignment of the published promoter regions of 19 *H. pylori* strains G. L. Narayanan (2005) identified a conserved stem loop structure which started at the +4 position of the mRNA transcript. Deletion of this stem loop

resulted in a reduction in *vacA* mRNA levels compared to the control. The alignment also revealed that this stem loop was well conserved except for differences at the +11 and +28 positions within the stem loop. Although the importance of these polymorphisms in determining *vacA* transcript level was not determined. Conversely the 5' stem loop structures appeared to have no affect on *vacA* transcription as no difference in *vacA* expression was observed when the whole 5' end of the untranslated region of *vacA* from Tox- strain 86-313 was replaced with that of Tox+ strain 60190 during the promoter exchange experiments by Forsyth *et al.,* (1999).

An inverted repeat upstream of the -35 motif was found to be present in 4 out of 8 clinical strains (Ayala *et al.,* 2004). Inverted repeats are a common feature of regulator binding sites, however the role of the inverted repeat in the *cysS* – *vacA* intergenic region in transcriptional regulation of *vacA* is yet to be determined.

4.2 Aims

Chapter 3 assessed the association between the level of *vacA* transcribed by an infecting strain with different disease parameters, showing that the level of *vacA* transcribed by an infecting strain *in vivo* is associated with severity of inflammation, activity and atrophy. Previous studies on *vacA* transcription have been carried out *in vitro*. They have led to the identification a *vacA* promoter, a stem loop structure at the 5' end of the *vacA* mRNA transcript and an inverted repeat upstream of the -35 motif. These regions have been shown to be

naturally polymorphic between strains, however no associations between these differences and the level of *vacA* mRNA has been found *in vivo* or *in vitro* (Forsyth *et al*., 1998, Forsyth and Cover, 1999, Ayala *et al*., 2004, Narayanan, 2005). The aims of this chapter are:

- To determine whether naturally occurring polymorphic differences in the *cysS* – *vacA* intergenic region contribute to different *vacA* transcriptional levels between clinical strains *in vivo.*
- To determine whether *vacA* transcript level *in vivo* correlates with the *vacA* transcript level in the same strains *in vitro* using matched strains and biopsies.
- To identify other potential binding sites for activators or repressors of transcription or structures that may affect mRNA stability; and to determine whether polymorphisms here are associated with differences in *vacA* transcription.
- To determine pattern of *vacA* transcription throughout growth of clinical strains *in vitro.*
- To confirm that differences in *vacA* transcription between strains are not due to "readthrough" from *cysS*.

4.3 Results

4.3.1 Characterisation of the effect of natural polymorphisms in the *cysS – vacA* intergenic region of clinical *H. pylori* strains *in vivo*.

To determine the sequence of the *cysS* – *vacA* intergenic region, clinical *H. pylori* strains were isolated and cultured from gastric biopsies taken from the corpus as detailed in section 2.4.4. Genomic DNA was extracted from *H. pylori*

strains using the methodology detailed in section 2.5.1. A 535 bp region including 47 bp of *cysS*, and a 159 bp region encoding the mature VacA cytotoxin was PCR-amplified from genomic DNA of the clinical *H. pylori* strains using the forward primer DL1 and the reverse primer VacR10. Both strands were sequenced using an Applied Biosystems 3100 Genetic analyser (Biopolymer Synthesis and Analysis unit, University of Nottingham, UK). Sequences retrieved were aligned using the MegAlign program (DNAstar), and ranked in order of levels of *vacA* mRNA/ 16s rRNA determined using real-time PCR data from chapter 3. The significance of the association between differences in transcript levels between strains and different bases present within the *vacA* promoter was analysed using the Mann-Whitney U test.

Analysis of the *cysS*-*vacA* intergenic region highlighted many polymorphic differences within the *vacA* promoter (Figure 4.1). Naturally occurring differences within the -35, -10 and the extended -10 motifs were identified. In agreement with previous studies on the *vacA* promoter, *in vitro* polymorphic differences were seen at position -32 within the -35 motif where there was a frequent G to A substitution. A G to A substitution was also found at position -14 within the extended -10 motif. However differences in the *vacA* transcript level between the different strains were not significantly associated with the polymorphic differences present at these sites.

Inverted repeats upstream of the -35 motif identified previously by Ayala *et al.*, (2004) were seen in 2 out of 27 strains, however the presence of an inverted repeat was unrelated to *vacA* mRNA levels *in vivo* in this study.

A stem loop structure beginning at the +4 site of the mRNA transcript has been identified previously (Narayanan, 2005). A G to A substitution was identified at position +11, and adenine to guanine and thymine to cytosine substitutions were seen at positions +17 and +18 respectively. Moreover these were not associated with the level of *vacA* transcript *in vivo*. However interestingly, strains possessing an A at position +28 within the second stem of the stem loop expressed more *vacA* than those that have a G at this position (P = 0.0089) (Figure 4.1.).

```
                Inverted Repeat                                                                                                                  -35
C A G C C A T T T T A C T T T - T T A A T C C T G T T A A G T T T T A - T A T T
                          10                                  20                                  30                                  40
. . A T A T . . . . G . . . . - . . . . . . . T . . . . - . G A . . . . . - . . . . . 194   30.22   s1m2i1
. . . . . . . G . . G . . . . - . . . . . . . T . . . . . . . . . . G . - . G . . 187   24.65   s1m1i1
. . . . . . . G . . G . . . . - . . . . . . . . . A . - G G . . . . . . - . . . . . 242   21.67   s2m2i2
. . . . A . . . . . . . . . . - . . . . . . . . . A . - G G . . . . . . - . . . . . 126   7.929   s1m2i1
T . . . A . . A . . G . . . . - . . . . . . . T . A . . . . . . . . C . - C . . . . 206   6.012   s1m1i1
. . . . . . . G . . . . . . . - . . . . . . . T . . . G . . T . . A . . . - . . C 255   5.110   s1m1i1
. . . . . . . . . . . . . . . - . . . . . . . . . A . - G G . . . . . . - . . . . . 249   3.842   s1m2i1
. . . . A . . . . . . . . . . - . . . . . . . . . A . - G G . . . . . . - . . . . . 256   1.900   s2m2i2
T . . T A . . A . . G . . . . - . . . . . . . T . . . . . . . . . . . . . - . G . . 253   1.521   s1m1i1
. . . . A T . . . . G . . . . - . . . . . - . T . . . . . . . . . . . . . - . . . . . 45    0       s1m1i1
. . . . A T . . . . G . . . . - . . . . . . . T . A . . . . . . . . . . . - . G . . 46    0       s1m1i1
. . . . . . . . . . . . . . . - . . . . . . . . . A . - G G . . . . . . - . . . . . 72    0       s1m1i1
. . . . . . . A . . G . . . . - . . . . . . . . . . . . G . . . . . . . - . . . . . 73    0       s1m2i2
. . . . . . . . . . . . . . . - . . . . . . . . . A . - G G . . . . . . - . . . . . 77    0       s1m1i1
. . . . . . . G . . G . . . . - . . . . . . . T . . . . G . . . . . . . - . G . . 83    0       s1m1i1
. . . . . . . A . . . . . . . - . . . . . . . . . A . - G T . . . . . . - . G . . 86    0       s1m1i1
. . . . . . . A . . . . . . . - . . . . . . . . . . . . . . . . . . . . - . G . . 120   0       s1m1i1
T . . . . . . A . . G . . . . - . . . . . . . T . . . . . . . . . . C . - C G . . 121   0       s1m1i1
. . . . . . . A . . . . . . . - . . . . . . . . . A . - G G . . . . . . - . . . . . 122   0       s1m1i2
. . . . A . . . . . . . . . . - . . . . . . . . . . . . . . . . . . . . - . G . . 124   0       s1m1i1
. . . . . . . A . . . . . . . - . . . . . . . . . . . . . . . . . . . . - . . . . . 181   0       s1m2i2
. . . . A T . . . . G . . . . - . . . . . . . T . . . . . . . . . . . . - . T . . 182   0       s1m1i1
T . . . A . . A . . G . . . . - . . . . . . . T . . . . G . . . . . . . - . T . . 192   0       s1m1i1
. . . . . . . A . . . . . . . - . . . . . . . . . . . . . . . . . . . . - . G . . 201   0       s1m1i1
. . . . A T . . . . G . . . . - . . . . . . . T . . . . . . . . . . . . - . G . . 218   0       s1m1i1
. . . . . . . A . . . . . . . . A . . . . . . . . . . . . . . . . . . . A . G . . 221   0       s1m2i2
. . . . A . . . . . . . . . . - . . . . . . . . . A . - G G . . . . . . - . . . . . 233   0       s2m2i2
```

Figure 4.1 Comparison of the *cysS – vacA* intergenic regions of clinical strains *in vivo*. The *cysS-vacA* intergenic regions were sequenced using the DL1 and VacR10 primers using an Applied Biosystems 3100 Genetic analyser (Biopolymer Synthesis and Analysis unit, University of Nottingham, UK). Sequences retrieved were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. The upstream inverted repeat and the -35 motif are highlighted in blue and green respectively. Perfect inverted repeats are highlighted in pink. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

```
                                                Extended
                                                -10        -10                  +1        Stem 1
C A T T T A T C T T A - A C T T G A T A A A A - A T T G A A C A T T G G T T G T
                                                                                                          Strain  vacA titre  vacA type
                         50                     60                      70                      80
. . . . . . C . . A . T . . . . . . . . . . . - - . . A . . . . . . C . . . . . .   194   30.22      s1m2i1
T . . . . . C . . . . . - . T . . . . . . . . . . . . . . . . . . . . . . . . . .   187   24.65      s1m1i1
. . . . . . . . . . . . . - . . . . A . . . . . . . . . . . . . . . . . C . . . . .   242   21.67      s2m2i2
. . . . . . . . . . . . . - . . . . A . . . . . . . . . . . . . . . . . . . . . . .   126   7.929      s1m2i1
T . . . . . C . . . . . - . T . . . . . . . . . . G . . T . . . . . . . . . . . . .   206   6.012      s1m1i1
T . . . . . C T . . . . - . T . . . . . . . . . . . . . . . . . T . . . . . . . . .   255   5.110      s1m1i1
. . . . . . . . . . . . . - . . . . A . . . . . . . . . . . . . . . . . C . . . . .   249   3.842      s1m2i1
. . . . . . . . . . . . . - . . . . A . . . . . . . . . . . . . . . . . . . . . . .   256   1.900      s2m2i2
T . . . . . C . . . . . - . T . . . . . . . . . . G . . T . . . . . . . . . . . . .   253   1.521      s1m1i1
. . . . . . . . . . . . . - . . . . . . . . . . . . . . . . . . . . . T . . . . . .   45    0          s1m1i1
. . . . . . C T . . . . - . T . . . . . . . . . . . . . . . . . . . . T . . . . . .   46    0          s1m1i1
. . . . . . . . . . . . . - . . . . . . . . . . . . . . . . . . . . . . . . . . . .   72    0          s1m1i1
. . . . . . . . . . . . . - . . . . . . . . . . . . . . . . . . . . . . . . . . . .   73    0          s1m2i2
. . . . . . . . . . . . . - . . . . . . . . . . . . . . . . . . . . . . . . . . . .   77    0          s1m1i1
. . . . . . C . . . . . - . T . . . . . . . . . . . . . . . . . . . . . . . . . . .   83    0          s1m1i1
. . . . . . . . . . . . . - . . . . A G . . . . . . . . . . . . . . . . . . . . . .   86    0          s1m1i1
. . . . . . C T . . . . - . T . . . . . . . . . . . . . . . . . . . . C . . . . . .   120   0          s1m1i1
T . . . . . C . . . . . - . T . . . . . . . . . . G . . T . . . . . . . . . . . . .   121   0          s1m1i1
. . . . . . C . . . . . - . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   122   0          s1m1i2
T . . . . . C . . . . . - . T . . . . . . . . . . . . . . T . . . . . . . . . . . .   124   0          s1m1i1
. . . . . . . . . . . . . - . . . . . . . . . . . . . . . . . . . . A . . . . . . .   181   0          s1m2i2
. . . . . . C T . . . . - . T . . . . . . . . . . . . . . . . . . . . T . . . . . .   182   0          s1m1i1
. . . . . . C T . . . . - . T . . . . . . . . T T . . . . . . . . . . T . . . . . .   192   0          s1m1i1
. . . . . . . . . . . . . - . . . . . . . . . T . . - . . . . . . . . . . . . . . .   201   0          s1m1i1
. . . . . . . . . . . . . - . . . . . . . . . . . . - . . . . . . . . . . T . . . .   218   0          s1m1i1
. . . . . . . . . . . . . - . . . . . . . . . G . . - . . . . . . . . . . . . . . .   221   0          s1m2i2
. . . . . . . . . . . . . - . . . . A . . . . . . . - . . . . . . . . . . . . C . .   233   0          s2m2i2
```

Comparison of the *cysS – vacA* intergenic regions of clinical strains *in vivo*. The *cysS-vacA* intergenic regions were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. The -10 and extended -10 motifs are highlighted in pink, the +1 site in blue and, the 5' stem loop structure in orange. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

	Loop	Stem 2		Strain	*vacA* titre	*vacA* type
	A G A T A	C T A T A	T A T T T A T A G C C T T A A T C G T A A A T G C A A C A G			
		90	100 110 120			
. A . . .	. . . . .	. . . . . A . .	. . . . . A	194	30.22	s1m2i1
. . . . .	. . G C .	. . . . . A . .	. . . . .	187	24.65	s1m1i1
. . . . .	. . G . .	. . . . . A . .	. . . . .	242	21.67	s2m2i2
. . . . .	. . . . .	. . . .	. . . . .	126	7.929	s1m2i1
. . . . .	. . . . .	. . . . C . C . A . .	. . . . .	206	6.012	s1m1i1
. . . . .	. . . . .	. . . .	. . . . .	255	5.110	s1m1i1
. . . . .	. . . . .	. . . . . A . .	. . . . .	249	3.842	s1m2i1
. . . . .	. . G C .	. . . . . A . .	. . . . .	256	1.900	s2m2i2
. . . . .	. . . . .	. . . . C . C . A . .	. . . . .	253	1.521	s1m1i1
. . . . .	. . . . .	. . . .	. . . . .	45	0	s1m1i1
. A . . .	. . . . .	. . . .	. . . . .	46	0	s1m1i1
. . . . .	. . G C .	. . . . . A . .	. . . . .	72	0	s1m1i1
. . . . .	. . . . .	. . . .	. . . . .	73	0	s1m2i2
. . . . .	. . . . .	. . . .	. . . . .	77	0	s1m1i1
. . . . .	. . . . .	. . . .	. . . . . A	83	0	s1m1i1
. . . . .	. . . . .	. . . .	. . . . .	86	0	s1m1i1
. . . . .	. . . . .	. . . .	. . . . .	120	0	s1m1i1
. . . . .	. . . . .	. . . . . A . .	. . . . .	121	0	s1m1i1
. . . . .	. . . . .	. . . .	. . . . . T	122	0	s1m1i2
. . . . .	. . . . .	. . . . C . C . A . .	. . . . .	124	0	s1m1i1
. . . . .	. . . . .	. . . .	. . . . .	181	0	s1m2i2
. . . . .	. . . . .	. . . .	. . . . . A	182	0	s1m1i1
. A . . .	. . . . .	. . . .	. . . . .	192	0	s1m1i1
. . . . .	. . G C .	. . . .	. . . . .	201	0	s1m1i1
. . . . .	. . . . .	. . . .	. . . . .	218	0	s1m1i1
. . . . .	. . G C .	. . . .	. . . . .	221	0	s1m2i2
. . . . .	. . . . .	. . . . . A . .	C	233	0	s2m2i2

Comparison of the *cysS – vacA* intergenic regions of clinical strains *in vivo*. The *cysS-vacA* intergenic regions were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. The 5' stem loop structure is highlighted in orange and the +28 position is highlighted in green. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

A A A T T T T C T A G T C T A A A G T C G C A C C C T T T G T G - - - - - C A A

130	140	150	160	Strain	*vacA* titre	*vacA* type
. . . . . C . . . T . G	. . . . .	. . . . .	- - - - - - . . .	194	30.22	s1m2i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	187	24.65	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	242	21.67	s2m2i2
. . . . .	. . . . .	. . . . .	- - - - - - . . .	126	7.929	s1m2i1
. . . . .	. . . . . G	. . . . .	- - - - - - . . .	206	6.012	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	255	5.110	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	249	3.842	s1m2i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	256	1.900	s2m2i2
. . . . .	. . . . .	. . . . . T . . .	A . . T T G T G . . .	253	1.521	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	45	0	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	46	0	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	72	0	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	73	0	s1m2i2
. . . . . T	. . . . .	. . . . .	- - - - - - . . .	77	0	s1m1i1
. . . . . T	. . . . .	. . . . .	- - - - - - . . .	83	0	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	86	0	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	120	0	s1m1i1
. . . . .	. . . . .	. . . . .	T T G T G . . .	121	0	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	122	0	s1m1i2
. . . . .	. . . . .	. . . . .	- - - - - - . . .	124	0	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	181	0	s2m2i2
. . . . .	. . . . .	. . . . .	- - - - - - . . .	182	0	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	192	0	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - - . . .	201	0	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - T G .	218	0	s1m1i1
. . . . . T	. . . . .	. . . . .	- - - - - - . . .	221	0	s1m2i2
. . . . .	. . . . .	. . . . . T . . .	- - - - - - . . .	233	0	s2m2i2

Comparison of the *cysS – vacA* intergenic regions of clinical strains *in vivo*. The *cysS-vacA* intergenic regions were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

```
                                                                                SD                TSP
A A A - T C G T T T T A C - A A A A A G A - - - - - - A A G G A A A A A A A T G
                                                                                                                  vacA   vacA
                    170                 180                 190                 200   Strain  titre  type
. . . - . . . . . . . . T T . . G . . . . - - - - - - . . . . . . G . . . . . .   194   30.22  s1m2i1
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . G . . . . . .   187   24.65  s1m1i1
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . G . . . . . .   242   21.67  s2m2i2
. . . A . T . . . . . . . - . . G . . . . - - - - - - . . . . . . . G . . . . .   126   7.929  s1m2i1
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . G . . . . . .   206   6.012  s1m1i1
. . . - . . . . . . . . - . . G . . . . - - - - - - . . . . . . . G . . . . .   255   5.110  s1m1i1
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . G . . . . . .   249   3.842  s1m2i1
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . G . . . . . .   256   1.900  s2m2i2
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . G . . . . . .   253   1.521  s1m1i1
. . . - . . . . . . . . - . . G . . . . - - - - - - . . . . . . . G . . . . .   45    0      s1m1i1
. . . - . . . . . . . . - . . G . . . . - - - - - - . . . . . . . G . . . . .   46    0      s1m1i1
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . G . . . . . .   72    0      s1m1i1
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . G . . . . . .   73    0      s1m2i2
. . . - . . . . . . . . - . . G . . . . T - - - - - . . . . . . . G . . . . .   77    0      s1m1i1
. . . - . . . . . . . . - . . G . . . . - - - - - - . . . . . . . G . . . . .   83    0      s1m1i1
. . . - . . . . . . . . - . . G . . . . - - - - - - . . . . . . . . . . . . .   86    0      s1m1i1
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . . G . . . . .   120   0      s1m1i1
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . G . . . . . .   121   0      s1m1i1
. . . - . . . . . . . . - . . G . . . . - - - - - - . . . . . . . . . . . . .   122   0      s1m1i2
. . . - . . . . . . . . - . . G . . A . G A A T A C . . . . . . . G . . . . .   124   0      s1m1i1
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . . G . . . . .   181   0      s1m2i2
. . . - . . . . . . . . - . . G . . . . - - - - - - . . . . . . G . . . . . .   182   0      s1m1i1
. . . - . T . . . . . . . - . . . . . . . - - - - - - . . . . . . G . . . . . .   192   0      s1m1i1
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . . G . . . . .   201   0      s1m1i1
. . . - . . . . . . . . T - . . . . . . . - - - - - - . . . . . . . . . . . . .   218   0      s1m1i1
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . . G . . . . .   221   0      s1m2i2
. . . - . . . . . . . . - . . . . . . . - - - - - - . . . . . . G . . . . . .   233   0      s2m2i2
```

Comparison of the *cysS – vacA* intergenic regions of clinical strains *in vivo*. The *cysS-vacA* intergenic regions were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. The Shine-Dalgarno sequence and the ATG start site are highlighted in blue and red respectively. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

G A A A T A C A A C A A A C A C A C C G C A A A A T C A A T C G C C C T C T G G

210	220	230	240	Strain	*vacA* titre	*vacA* type
. . . . .	. . . . .	. . . . .	T . A .	194	30.22	s1m2i1
. . . . .	. . . . .	. . . . .	T . . .	187	24.65	s1m1i1
. . . . .	. . . . .	. . . . .	A . T A	242	21.67	s2m2i2
. . . . .	. . . . .	. . . . .	. . . .	126	7.929	s1m2i1
. . . . .	. . . . . G	. . . . .	T . A .	206	6.012	s1m1i1
. . . . .	. . . . .	. . . . . T	. . . . .	255	5.110	s1m1i1
. . . . .	. . . . .	. . . . .	. . . .	249	3.842	s1m2i1
. . . T	. . . . .	. . . . .	A . T A	256	1.900	s2m2i2
. . . T	. . . . . G	. . . . .	T . A .	253	1.521	s1m1i1
. . . . .	. . . . . G	. . . . .	T . A .	45	0	s1m1i1
. . . . .	. . . . .	. . . . .	. . . .	46	0	s1m1i1
. . . . .	. . . . .	. . . . .	. . . .	72	0	s1m1i1
. . . T	. . . . .	. . . . .	. . . .	73	0	s1m2i2
. . . . .	. . . . .	. . . . .	T . A .	77	0	s1m1i1
. . . . .	. . . . .	. . . . .	. . . .	83	0	s1m1i1
. . . . .	. . . . .	. . . . .	T . . .	86	0	s1m1i1
. . . . .	. . . . .	. . . . .	T . . .	120	0	s1m1i1
. . . . . G	. . . . .	. . . . . G	. . . . .	T . A .	121	0	s1m1i1
. . . . .	. . . . .	. . . . .	T . A .	122	0	s1m1i2
. . . . .	. . . . .	. . . . . T	. . . T	. . .	124	0	s1m1i1
. G	. . . . .	. . . . .	T . . .	181	0	s1m2i2
. . . . .	. . . . .	. . . . .	. . . .	182	0	s1m1i1
. . . T	. . . . .	. . . . .	. . . .	192	0	s1m1i1
. . . . .	. . . . .	. . . . .	. . . .	201	0	s1m1i1
. . . . .	. . . . .	. . . . .	. . . .	218	0	s1m1i1
. . . . .	. . . . .	. . . . .	. . . .	221	0	s1m2i2
. . . . .	. . . . .	. . . . .	A . T A	233	0	s2m2i2

Comparison of the *cysS – vacA* intergenic regions of clinical strains *in vivo*. The *cysS-vacA* intergenic regions were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

```
T T T C T C T C G C T T T A G T A G G
                    250
                                                  Strain  vacA  vacA
                                                          titre  type
. . . . . . . . . . . . . . . . . G . .  194  30.22  s1m2i1
. . . . . . . . . . . . . . . . . . . .  187  24.65  s1m1i1
. C . . . . . . . . . C . . . . G . .  242  21.67  s2m2i2
. . . . . . . T . . . . . . . . . . . .  126  7.929  s1m2i1
. . . . . . . . . T . . . . . C . . .  206  6.012  s1m1i1
. . . . . . . . . . . . . . . . . . . .  255  5.110  s1m1i1
. . . . . . . . . . . . . . . . . . . .  249  3.842  s1m2i1
. C . . . . . . . . . . . . . . . G . .  256  1.900  s2m2i2
. . . . . . . . . T . . . . . C . . .  253  1.521  s1m1i1
. . G . . . . . . T . . . . . C . . .  45   0      s1m1i1
. . . . . . . T . . . . . . . . . . . .  46   0      s1m1i1
. . . . . . . T . . . . . . . . . . . .  72   0      s1m1i1
. . . . . . . T . . . . . . . . . . . .  73   0      s1m2i2
. . . . . . . . . . . . . . . . . . . .  77   0      s1m1i1
. . . . . . . . . . . . . . . . . . . .  83   0      s1m1i1
. . . . . . . . . . . . . . . . . . . .  86   0      s1m1i1
. . . . . . . . . . . . . . . . . . . .  120  0      s1m1i1
. . . . . . . . . T . . . . . C . . .  121  0      s1m1i1
. . . . . . . . . T . . . . . C . . .  122  0      s1m1i2
. . . . . . . T . . . . . . . . . . . .  124  0      s1m1i1
. . . . . . . . . . . . . . . . . . . .  181  0      s1m2i2
. . . . . . . T . . . . . . . . . . . .  182  0      s1m1i1
. . . . . . . T . . . . . . . . . . . .  192  0      s1m1i1
. . . . . . . T . . . . . . . . . . . .  201  0      s1m1i1
. . . . . . . . . . . . . . . . . . . .  218  0      s1m1i1
. . . . . . . T . . . . . . . . . . . .  221  0      s1m2i2
. C . . . . . . . . . . . . . . . G . .  233  0      s2m2i2
```

Comparison of the *cysS – vacA* intergenic regions of clinical strains *in vivo*. The *cysS-vacA* intergenic regions were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

4.3.2 Characterisation of the effect of natural polymorphisms in the *cysS – vacA* intergenic region of clinical *H. pylori* strains *in vitro*.

Previous studies have investigated the heterogeneity between *vacA* transcript levels between strains *in vitro*. To determine whether there is a correlation between *vacA* mRNA levels produced by strains in the gastric mucosa *in vivo* and in the same *H. pylori* strains grown *in vitro* strains were isolated from corporal gastric biopsies and cultured and their *vacA* mRNA level assessed by real-time PCR.

4.3.3 Quantitative real-time PCR using RNA extracted from broth cultures.

In order to quantify the amount of *vacA* transcript present per *H. pylori* cell in broth culture, RNA was extracted from *H. pylori* broth culture of strain 60190 as described in section 2.6.1. Total RNA was quantified using the Nanodrop spectrophotometer. RNA samples were adjusted to 33.3ng/µl to normalise the concentration of RNA for subsequent DNAfree treatment and cDNA synthesis. DNA contamination was removed using a DNAfree kit (Ambion) as described in section 2.6.4. cDNA was synthesised using 1µg of total RNA using the Omniscript RT kit (Qiagen, UK) as described in section 2.6.5. A minus reverse transcriptase containing the cDNA master mix and RNA sample without reverse transcriptase was also produced for each sample to confirm the absence of residual DNA in the RNA samples.

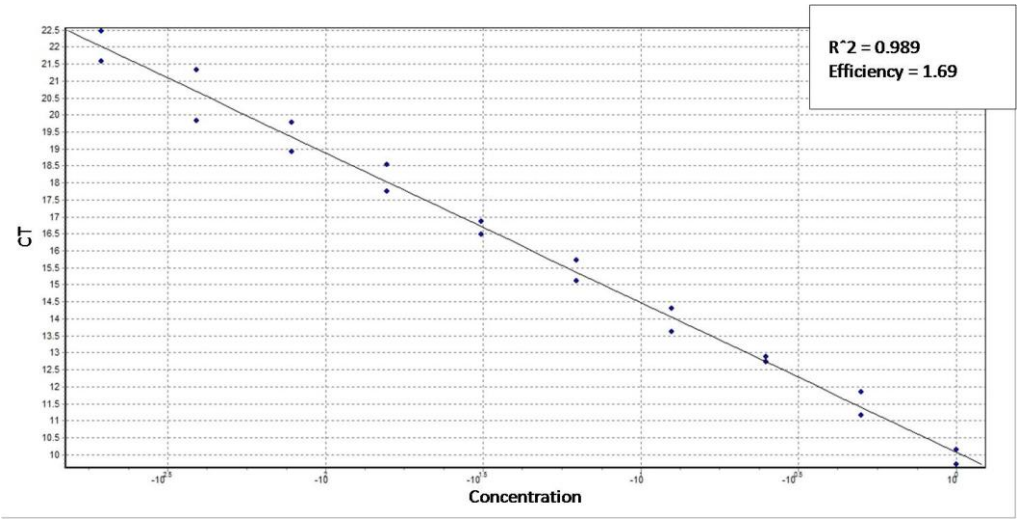
Quantitative real-time PCR was performed on the Rotorgene 3000 using the methodology outlined in section 2.6.6. Differences in *vacA* mRNA levels between strains were assessed using the Pfaffl method of analysis. The level of *vacA* transcript per *H. pylori* cell in all the broth samples tested was compared to that of strain 60190, which was included in triplicate as a control in each real-time PCR run to control for inter-experimental variations.

To determine the amplification efficiency of both *vacA* and 16s rRNA a sample of control cDNA (60190) was serially diluted in triplicate to produce a standard curve (Figure 4.2). The amplification efficiencies were calculated from the slopes of the standard curve according to the equation E = $10^{[10-1/slope]}$. The amplification efficiencies for *vacA* and 16s rRNA were 1.69 (Figure 4.2 (A)) and 1.88 (Figure 4.2 (B)) respectively, with R^2 values of 0.989 and 0.988 respectively.

The optimised real-time PCR was performed in triplicate for each broth culture tested; the mean C_T value was used to calculate the relative expression of *vacA*. The level of *vacA* mRNA per *H. pylori* cell was expressed relative to sample 60190. Blanks containing the real-time PCR master mixes for both the *vacA* and 16s rRNA primers were tested on each run to confirm the absence of contamination in the master mix.

Melt curves for all samples tested, including the cDNA negative and blanks were checked on every run to confirm the absence of contamination (Figure 4.3).

A



B

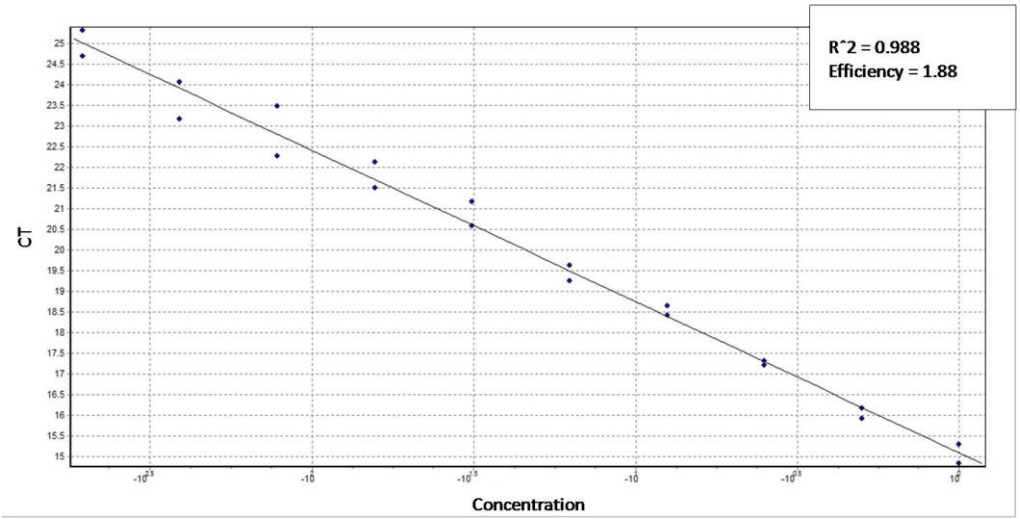


Figure 4.2 Standard curve and amplification efficiency of 16S (A) and *vacA* (B). To determine the amplification efficiencies of 16S and *vacA* using templates extracted from *H. pylori* broth culture, a sample of control cDNA 60190 was serially diluted to produce a standard curve. The amplification efficiencies were calculated from the slopes of the standard curve according to the equation E = $10^{[10-1/slope]}$. The amplification efficiencies for *vacA* and 16S rRNA were 1.88 and 1.69 respectively, with R2 values of 0.988 and 0.989 respectively.

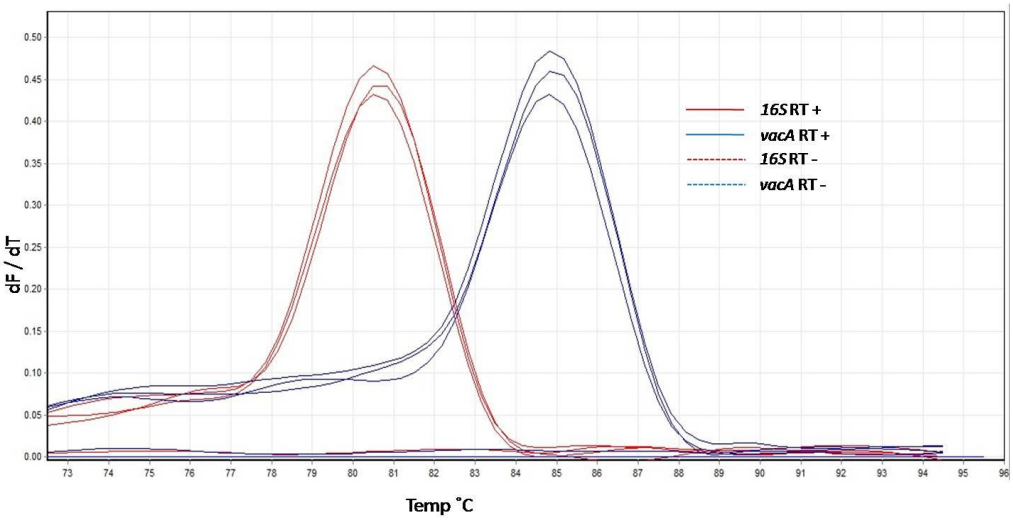


Figure 4.3 Melt curves of 16S and *vacA* PCR products synthesised from template extracted from broth culture. The melt curve shows the rate of change of relative fluorescence units (RFU) with time (T) (d-(RFU)/dT) on the Y axis against the temperature on the x axis. The melt curves are performed at the end of the amplification reactions to determine the melting temperature of the PCR product. The single peak in each melt curve indicates that only one product is present for each primer pair. The lack of peak in the RT – reactions also indicates that no contamination or primer dimer artefacts were present.

4.3.4 Assessment of *vacA* transcript levels throughout the growth curves of *H. pylori* clinical strains

Microarray studies and RNA dot blots have shown that *vacA* transcription appears to peak at late log to early stationary phase (Thompson *et al.,* 2003, Narayanan 2005). To determine the growth profile of the clinical strains grown using the culture conditions in this study four clinical strains cultured on blood agar plates were used to inoculate 30 ml of SFBB supplemented with 5% FBS at a starting OD_{600} of 0.1. Cultures were contained in a $75cm^3$ tissue culture flask and grown with shaking (100rpm) at 37˚C in an atmosphere of 5% CO_2 and 80% humidity for 30 hours. Broth samples were taken at time points 0, 3, 6, 9, 11, 12, 15, 18, 24.75, 27 and 29.5 hours, and the OD_{600} noted. Broth cultures were set up in triplicate for each clinical strain and samples were routinely Gram stained to confirm the absence of contamination. The growth profiles of the four clinical *H. pylori* strains show that although some of the strains grew to higher optical densities that others, all strains entered stationary phase at approximately 18 hours (Figure 4.4).

Previous studies have shown that *vacA* transcription appears to peak at the switch between the exponential and stationary phase in laboratory strains. To assess whether this is the case in the clinical strains used in this study, and to be sure that the differences in *vacA* expression seen between strains *in vitro* would not be due to slight differences in growth, samples were taken for RNA extraction from the broth cultures detailed above at time points 15, 18 and 21, hours which correlated to late log or early stationary phase. One sample was taken for RNA extraction from each broth for each time point (i.e. 3 broths per

strain per time point). The level of *vacA* mRNA per bacterial cell was assessed by real-time PCR detailed in section 4.3.3 and was analysed using the Pfaffl method (Pfaffl, 2001). The level of *vacA* mRNA per cell was expressed relative to sample 60190 which was used as a control on every real-time PCR run. *vacA* mRNA expression did not differ significantly between the 15, 18 and 21 hour time points in all four clinical strains tested (Figure 4.5). Thus, samples of clinical strains were taken at 18 hours in subsequent experiments for quantitative real-time PCR analysis.

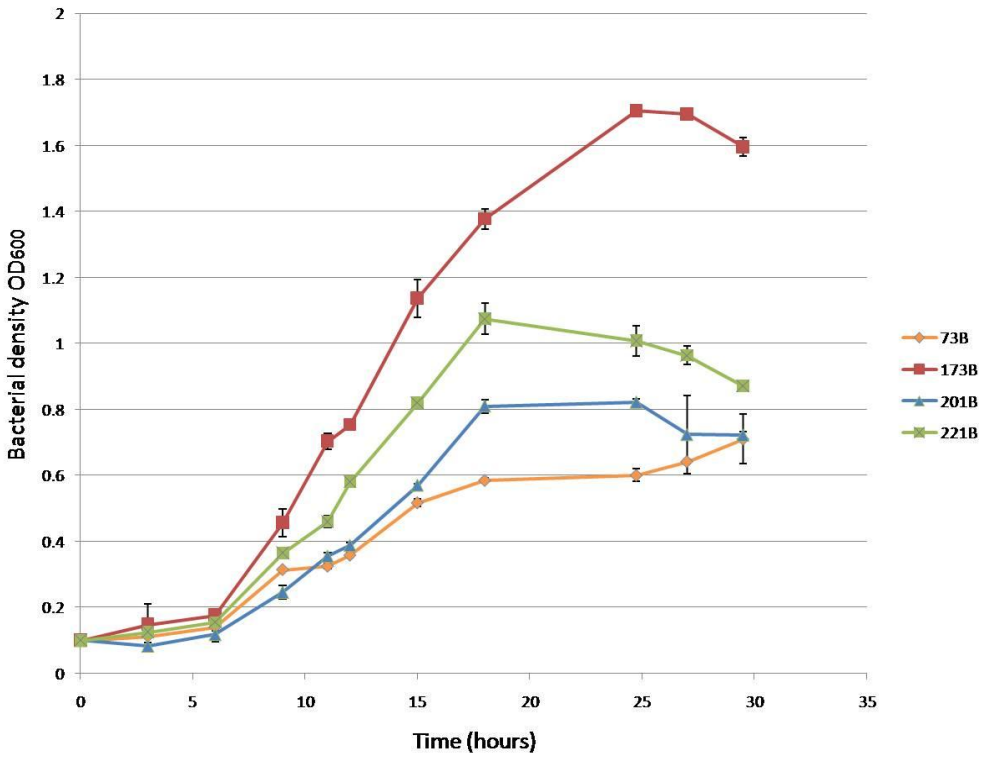


Figure 4.4 Growth profiles of clinical *H. pylori* strains 73B, 173B, 201B and 221B. The mean bacterial density (OD600) based on values from three separate broth cultures for each clinical strain was plotted against time (hours). Error bars show the standard deviation of the mean bacterial density at each time point.

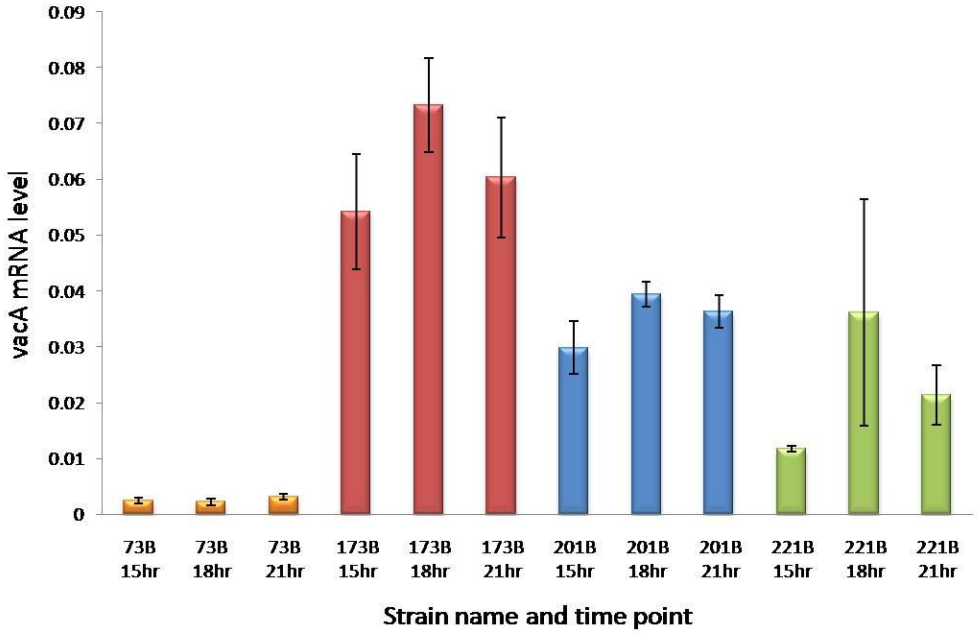


Figure 4.5 Comparison of *vacA* mRNA levels at 15, 18 and 21 hours in clinical *H. pylori* strains. The bar chart above shows the mean *vacA* mRNA level per *H. pylori* cell for clinical strains 73B, 173B, 201B and 221B at 15, 18 and 21 hours. The error bars show the standard deviation of the mean relative *vacA* mRNA level for each strain at each time point. The *vacA* mRNA level did not differ significantly between the 15, 18 and 21 hour time points in any strain tested.

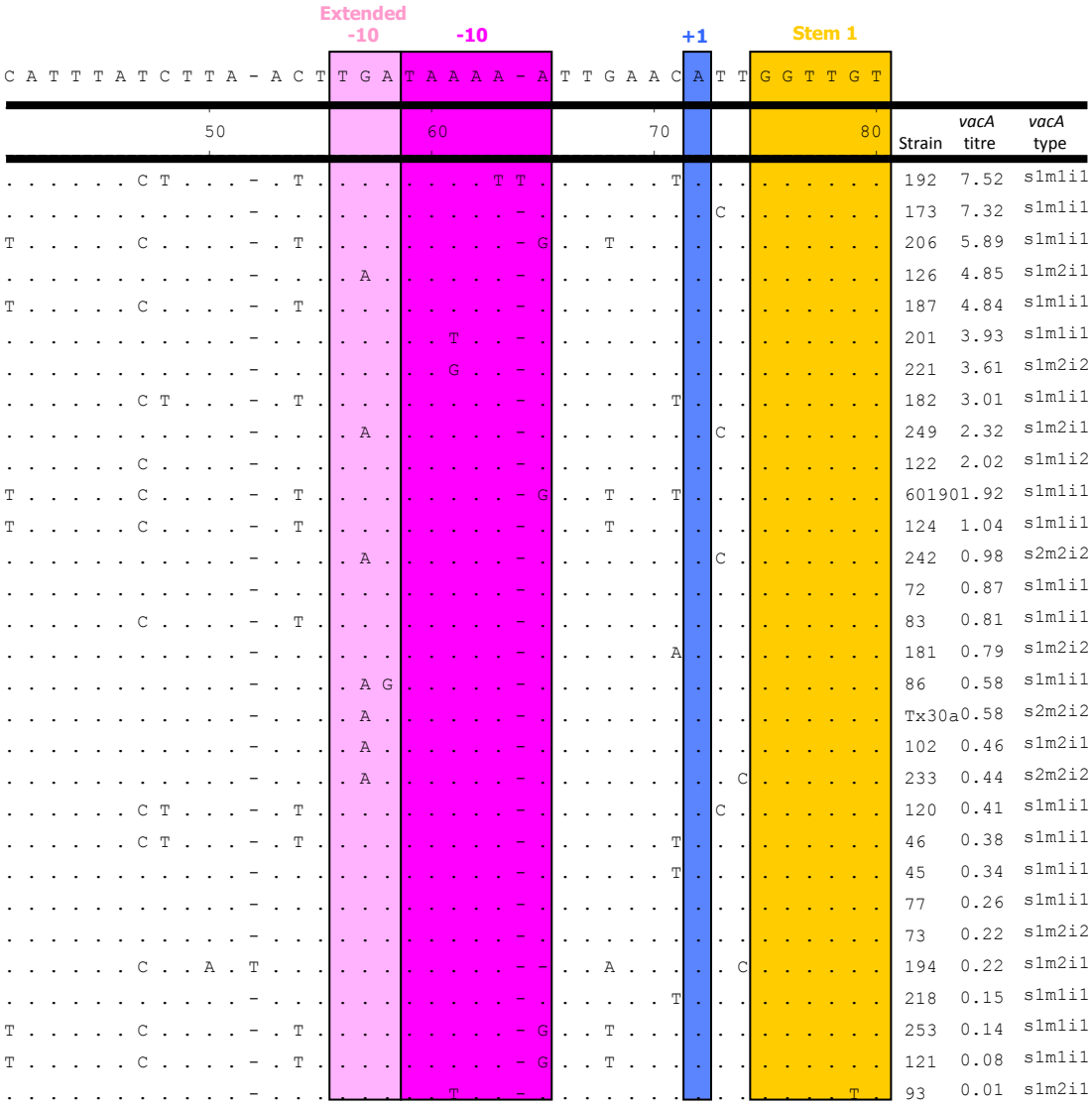
4.3.5 Characterisation of the effect of natural polymorphisms in the *cysS – vacA* intergenic region of clinical *H. pylori* strains *in vitro*.

Thirty clinical strains were grown in broth culture, samples were taken for RNA extraction at 18hrs, and *vacA* mRNA levels were assessed by real-time PCR as detailed previously in sections 4.3.3 and 4.3.4. Alignments of the *cysS – vacA* intergenic regions of the clinical strains shown in section 4.3.1 were ranked in order of detectable *vacA* mRNA level per *H. pylori* cell *in vitro*. Differences in transcript levels between strains with different polymorphisms present within the *vacA* promoter were assessed using the Mann-Whitney U test. Up to 180 fold differences in *vacA* expression were observed between strains. In agreement with previous studies the sequence alignment shows that some strains differ in the nucleotide present at the final position in the -35 region. Analysis of the clinical strains also shows that most strains possess a TGA extended -10 region but some strains possess a TAA motif. However the differences between strains in the -35 and extended -10 regions are not significantly associated with levels of *vacA* transcription. A potential region of interest is the inverted repeat upstream of the -35 motif identified by Ayala *et al.,* (2004). The sequence alignment reveals that strains with a fully palindromic inverted repeat express significantly more *vacA* than other strains (P = 0.029). Interestingly, polymorphic differences at position +28 of the stem loop at the beginning of the *vacA* mRNA transcript were not associated with levels of *vacA* mRNA *in vitro* in this study (Figure 4.6).

```
                Inverted Repeat                                                                                  -35
C A G C C A T T T T A C T T T - T T A A T C C T G T T G A G T T T T A - T A T T
                          10                  20                  30                                  40
T . . . A . . A . . G . . . . - . . . . . . T . . . . . . . . . . . . . . - . T . .   192   7.52   s1m1i1
. . . . . . . . . . . . . . - . . . . . . . . . A . - . G . . . . . . . . - . . . .   173   7.32   s1m1i1
T . . . A . . A . . G . . . . - . . . . . . T . A . . A . . . . . . C . - C . . . .   206   5.89   s1m1i1
. . . . A . . . . . . . . . - . . . . . . . . . A . - . G . . . . . . . . - . . . .   126   4.85   s1m2i1
. . . . . . . G . . G . . . . - . . . . . . T . . . . A . . . . . . G . - . G . . .   187   4.84   s1m1i1
. . . . . . . A . . . . . . . - . . . . . . . . . . . A . . . . . . . . - . G . . .   201   3.93   s1m1i1
. . . . . . . A . . . . . . . . A . . . . . . . . . . . A . . . . . . . . A . G . .   221   3.61   s1m2i2
. . . . A T . . . . G . . . . - . . . . . . T . . . . A . . . . . . . . - . T . . .   182   3.01   s1m1i1
. . . . . . . . . . . . . . . - . . . . . . . . . A . - . G . . . . . . . . - . . . .   249   2.32   s1m2i1
. . . . . . . A . . . . . . . - . . . . . . . . . A . - . G . . . . . . . . - . . . .   122   2.02   s1m1i2
T . . . A . . A . . G . . . . - . . . . . . T . . . . . . . . . . . . . . - . G . .   60190 1.92   s1m1i1
. . . . A . . . . . . . . . . - . . . . . . . . . . . A . . . . . . . . - . G . . .   124   1.04   s1m1i1
. . . . . . . G . . G . . . . - . . . . . . . . . A . - . G . . . . . . . . - . . . .   242   0.98   s2m2i2
. . . . . . . . . . . . . . . - . . . . . . . . . A . - . G . . . . . . . . - . . . .   72    0.87   s1m1i1
. . . . . . . G . . G . . . . - . . . . . . T . . . . . . . . . . . . . . - . G . .   83    0.81   s1m1i1
. . . . . . . A . . . . . . . - . . . . . . . . . . . A . . . . . . . . - . . . . .   181   0.79   s1m2i2
. . . . . . . A . . . . . . . - . . . . . . . . . A . - . T . . . . . . . . - . G . .   86    0.58   s1m1i1
. . . . . . . . . . . . . . . - . . . . . . . . . A . - . G . . . . . . . . - . . . .   Tx30a 0.58   s2m2i2
. . . . . . . . . . . . . . . - . . . . . . . . . A . - . G . . . . . . . . - . . . .   102   0.46   s1m2i1
. . . . A . . . . . . . . . . - . . . . . . . . . A . - . G . . . . . . . . - . . . .   233   0.44   s2m2i2
. . . . . . . A . . . . . . . - . . . . . . . . . . . A . . . . . . . . - . G . . .   120   0.41   s1m1i1
. . . . A T . . . . G . . . . - . . . . . . T . A . . A . . . . . . . . - . G . . .   46    0.38   s1m1i1
. . . . A T . . . . G . . . . - . . . . . - . T . . . . A . . . . . . . . - . . . .   45    0.34   s1m1i1
. . . . . . . . . . . . . . . - . . . . . . . . . A . - . G . . . . . . . . - . . . .   77    0.26   s1m1i1
. . . . . . . A . . G . . . . - . . . . . . . . . . . . . . . . . . . . . . - . . . .   73    0.22   s1m2i2
. . A T A T . . . . G . . . . - . . . . . . . T . . . - A G A . . . . . . . - . . . .   194   0.22   s1m2i1
. . . . A T . . . . G . . . . - . . . . . . . T . . . . A . . . . . . . . . - . G . .   218   0.15   s1m1i1
T . . T A . . A . . G . . . . - . . . . . . . T . . . . A . . . . . . . . . - . G . .   253   0.14   s1m1i1
T . . . . . . A . . G . . . . - . . . . . . . T . . . . A . . . . . C . - C G . . .   121   0.08   s1m1i1
. . . . . . . A . . . . . . . - . . . . . . . . . . . A . . . . . . . . - . . . . .   93    0.01   s1m2i1
```

Strain	*vacA* titre	*vacA* type
192	7.52	s1m1i1
173	7.32	s1m1i1
206	5.89	s1m1i1
126	4.85	s1m2i1
187	4.84	s1m1i1
201	3.93	s1m1i1
221	3.61	s1m2i2
182	3.01	s1m1i1
249	2.32	s1m2i1
122	2.02	s1m1i2
60190	1.92	s1m1i1
124	1.04	s1m1i1
242	0.98	s2m2i2
72	0.87	s1m1i1
83	0.81	s1m1i1
181	0.79	s1m2i2
86	0.58	s1m1i1
Tx30a	0.58	s2m2i2
102	0.46	s1m2i1
233	0.44	s2m2i2
120	0.41	s1m1i1
46	0.38	s1m1i1
45	0.34	s1m1i1
77	0.26	s1m1i1
73	0.22	s1m2i2
194	0.22	s1m2i1
218	0.15	s1m1i1
253	0.14	s1m1i1
121	0.08	s1m1i1
93	0.01	s1m2i1

Figure 4.6 Comparison of the *cysS – vacA* intergenic regions of clinical strains cultured *in vitro*. The *cysS-vacA* intergenic regions were sequenced using the DL1 and VacR10 primers using an Applied Biosystems 3100 Genetic analyser (Biopolymer Synthesis and Analysis unit, University of Nottingham, UK). Sequences retrieved were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. The upstream inverted repeat and the -35 motif are highlighted in blue and green respectively. Perfect inverted repeats are highlighted in pink. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.



Comparison of the *cysS* – *vacA* intergenic regions of clinical strains cultured *in vitro*. The *cysS-vacA* intergenic regions were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. The -10 and extended -10 motifs are highlighted in pink, the +1 site in blue and, the 5' stem loop structure in orange. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

	Loop	Stem 2		Strain	*vacA* titre	*vacA* type
AGATA	CTATA	TATTTATAGCC	TTAATCGTAAATGCAACAG			
.A...	.....	....	....	192	7.52	s1m1i1
.....	.....	....	....	173	7.32	s1m1i1
.....	.....	....C.C.A..	....	206	5.89	s1m1i1
.....	.....	....	....	126	4.85	s1m2i1
.....	..GC.	........A..	....	187	4.84	s1m1i1
.....	..GC.	....	....	201	3.93	s1m1i1
.....	..GC.	....	....	221	3.61	s1m2i2
.....	.....	....	............A......	182	3.01	s1m1i1
.....	.....	........A..	....	249	2.32	s1m2i1
.....	.....	....	.............T.....	122	2.02	s1m1i2
.....	..GC.	....	....	60190	1.92	s1m1i1
.....	.....	....C.C.A..	....	124	1.04	s1m1i1
.....	..G..	........A..	....	242	0.98	s2m2i2
.....	..GC.	........A..	....	72	0.87	s1m1i1
.....	.....	....	......A............	83	0.81	s1m1i1
.....	.....	....	....	181	0.79	s1m2i2
.....	.....	....	....	86	0.58	s1m1i1
.....	..GC.	........A..	....	Tx30a	0.58	s2m2i2
.....	..GC.	........A..	....	102	0.46	s1m2i1
.....	.....	........A..	C..................	233	0.44	s2m2i2
.....	.....	....	....	120	0.41	s1m1i1
.A...	.....	....	....	46	0.38	s1m1i1
.....	.....	....	....	45	0.34	s1m1i1
.....	.....	....	....	77	0.26	s1m1i1
.....	.....	....	....	73	0.22	s1m2i2
.A...	.....	........A..	............A......	194	0.22	s1m2i1
.....	.....	....	....	218	0.15	s1m1i1
.....	.....	....C.C.A..	....	253	0.14	s1m1i1
.....	.....	........A..	....	121	0.08	s1m1i1
.....	.....	....	....	93	0.01	s1m2i1

Comparison of the *cysS* – *vacA* intergenic regions of clinical strains cultured *in vitro*. The *cysS-vacA* intergenic regions were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. The 5' stem loop structure is highlighted in orange and the +28 position is highlighted in green. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

```
A A A T T T T C T A G T C T A A A G T C G C A C C C T T T G T G - - - - - C A A
```

130	140	150	160	Strain	*vacA* titre	*vacA* type
. . . . .	. . . . .	. . . . .	- - - - - . . .	192	7.52	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	173	7.32	s1m1i1
. . . . .	. . . . . G	. . . . .	- - - - - . . .	206	5.89	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	126	4.85	s1m2i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	187	4.84	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	201	3.93	s1m1i1
. . . . .	. . . . . T	. . . . .	- - - - - . . .	221	3.61	s1m2i2
. . . . .	. . . . .	. . . . .	- - - - - . . .	182	3.01	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	249	2.32	s1m2i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	122	2.02	s1m1i2
. . . . .	. . . . . T	. . . . .	- - - - - . . .	60190	1.92	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	124	1.04	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	242	0.98	s2m2i2
. . . . .	. . . . .	. . . . .	- - - - - . . .	72	0.87	s1m1i1
. . . . .	. . . . . T	. . . . .	- - - - - . . .	83	0.81	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	181	0.79	s1m2i2
. . . . .	. . . . .	. . . . .	- - - - - . . .	86	0.58	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	Tx30a	0.58	s2m2i2
. . . . .	. . . . .	. . . . .	- - - - - . . .	102	0.46	s1m2i1
. . . . .	. . . . .	. . . . . T	- - - - - . . .	233	0.44	s2m2i2
. . . . .	. . . . .	. . . . .	- - - - - . . .	120	0.41	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	46	0.38	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	45	0.34	s1m1i1
. . . . .	. . . . . T	. . . . .	- - - - - . . .	77	0.26	s1m1i1
. . . . .	. . . . .	. . . . .	- - - - - . . .	73	0.22	s1m2i2
. . . . . C . . . T . G	. . . . .	. . . . .	- - - - - . . .	194	0.22	s1m2i1
. . . . .	. . . . .	. . . . .	- - - - - T G .	218	0.15	s1m1i1
. . . . .	. . . . .	. . . . . T . . . A . .	T T G T G . . .	253	0.14	s1m1i1
. . . . .	. . . . .	. . . . .	T T G T G . . .	121	0.08	s1m1i1
. . . . .	. . . . .	. . . . . T	- - - - - . . .	93	0.01	s1m2i1

Comparison of the *cysS – vacA* intergenic regions of clinical strains cultured *in vitro*. The *cysS-vacA* intergenic regions were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

```
                                                                                SD                TSP
A A A - T C G T T T T A C - A A A A A G A - - - - - - A A G G A A A A A A T G
                    170                 180                 190                 200
. . . - . T . . . . . . . - . . . . . . . . - - - - - - . . . . . . G . . . . . . .   192   7.52  s1m1i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . G . . . . . . .   173   7.32  s1m1i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . G . . . . . . .   206   5.89  s1m1i1
. . . A . T . . . . . . . - . . G . . . . - - - - - - . . . . . . . . G . . . . . .   126   4.85  s1m2i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . G . . . . . . .   187   4.84  s1m1i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . . . G . . . . .   201   3.93  s1m1i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . . . G . . . . .   221   3.61  s1m2i2
. . . - . . . . . . . . . - . . G . . . . - - - - - - . . . . . . G . . . . . . .   182   3.01  s1m1i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . G . . . . . . .   249   2.32  s1m2i1
. . . - . . . . . . . . . - . . G . . . . - - - - - - . . . . . . . . . . . . . .   122   2.02  s1m1i2
. . . A . T . . . . . . . - . . G . . A . G A A G A - . . . . . . . . G . . . . . .   60190 1.92  s1m1i1
. . . - . . . . . . . . . - . . G . . A . G A A T A C . . . . . . . . G . . . . . .   124   1.04  s1m1i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . G . . . . . . .   242   0.98  s2m2i2
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . G . . . . . . .   72    0.87  s1m1i1
. . . - . . . . . . . . . - . - - - - - . G A A G A - . . . . . . . . G . . . . . .   83    0.81  s1m1i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . . . G . . . . .   181   0.79  s1m2i2
. . . - . . . . . . . . . - . . G . . . . - - - - - - . . . . . . . . . . . . . .   86    0.58  s1m1i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . G . . . . . . .   Tx30a 0.58  s2m2i2
. . . - . . . . . . . . . - . . G . . . . - - - - - - . . . . . . . . G . . . . . .   102   0.46  s1m2i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . G . . . . . . .   233   0.44  s2m2i2
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . . . G . . . . .   120   0.41  s1m1i1
. . . - . . . . . . . . . - . . G . . . . - - - - - - . . . . . . . . G . . . . . .   46    0.38  s1m1i1
. . . - . . . . . . . . . - . . G . . . . - - - - - - . . . . . . . . G . . . . . .   45    0.34  s1m1i1
. . . - . . . . . . . . . - . . G . . . . T - - - - - . . . . . . . . G . . . . . .   77    0.26  s1m1i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . G . . . . . . .   73    0.22  s1m2i2
. . . - . . . . . . . . T T . . G . . . . - - - - - - . . . . . . G . . . . . . .   194   0.22  s1m2i1
. . . - . . . . . . . . T - . . . . . . . . - - - - - - . . . . . . . . . . . . . .   218   0.15  s1m1i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . G . . . . . . .   253   0.14  s1m1i1
. . . - . . . . . . . . . - . . . . . . . . - - - - - - . . . . . . G . . . . . . .   121   0.08  s1m1i1
. . . - . . . . . . . . . - . . G . . . . - - - - - - . . . . . . . . . . . . . .   93    0.01  s1m2i1
```

Comparison of the *cysS* – *vacA* intergenic regions of clinical strains cultured *in vitro*. The *cysS-vacA* intergenic regions were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. The Shine-Dalgarno sequence and the ATG start site are highlighted in blue and red respectively. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

G A A A T A C A A C A A A C A C A C C G C A A A A T C A A T C G C C C T C T G G

210	220	230	240	Strain	*vacA* titre	*vacA* type
. . . T	. . . . .	. . . . .	. . . . .	192	7.52	s1m1i1
. . . . .	. . . . .	. . . . .	. . . . .	173	7.32	s1m1i1
. . . . .	. . . . .	G	. . . . . T . A .	206	5.89	s1m1i1
. . . . .	. . . . .	. . . . .	. . . . .	126	4.85	s1m2i1
. . . . .	. . . . .	. . . . .	. . . . . T . . .	187	4.84	s1m1i1
. . . . .	. . . . .	. . . . .	. . . . .	201	3.93	s1m1i1
. . . . .	. . . . .	. . . . .	. . . . .	221	3.61	s1m2i2
. . . . .	. . . . .	. . . . .	. . . . .	182	3.01	s1m1i1
. . . . .	. . . . .	. . . . .	. . . . .	249	2.32	s1m2i1
. . . . .	. . . . .	. . . . .	. . . . . T . A .	122	2.02	s1m1i2
. . . . .	. . . . .	. . . . .	. . . . .	60190	1.92	s1m1i1
. . . . .	. . . . .	. . . . .	T . . . . T . . .	124	1.04	s1m1i1
. . . . .	. . . . .	. . . . .	. . . . . A . T A	242	0.98	s2m2i2
. . . . .	. . . . .	. . . . .	. . . . .	72	0.87	s1m1i1
. . . . .	. . . . .	. . . . .	. . . . .	83	0.81	s1m1i1
. G	. . . . .	. . . . .	. . . . . T . . .	181	0.79	s1m2i2
. . . . .	. . . . .	. . . . .	. . . . . T . . .	86	0.58	s1m1i1
. . . . .	. . . . .	. . . . .	. . . . . A . T A	Tx30a	0.58	s2m2i2
. . . . .	. . . . .	. . . . .	. . . . .	102	0.46	s1m2i1
. . . . .	. . . . .	. . . . .	. . . . . A . T A	233	0.44	s2m2i2
. . . . .	. . . . .	. . . . .	. . . . . T . . .	120	0.41	s1m1i1
. . . . .	. . . . .	. . . . .	. . . . .	46	0.38	s1m1i1
. . . . .	. . . . .	G	. . . . . T . A .	45	0.34	s1m1i1
. . . . .	. . . . .	. . . . .	. . . . . T . A .	77	0.26	s1m1i1
. . . T	. . . . .	. . . . .	. . . . .	73	0.22	s1m2i2
. . . . .	. . . . .	. . . . .	. . . . . T . A .	194	0.22	s1m2i1
. . . . .	. . . . .	. . . . .	. . . . .	218	0.15	s1m1i1
. . . T	. . . . .	G	. . . . . T . A .	253	0.14	s1m1i1
. . . . . G	. . . . .	G	. . . . . T . A .	121	0.08	s1m1i1
. . . . .	. . . . .	. . . . .	. . . . . T . . .	93	0.01	s1m2i1

Comparison of the *cysS – vacA* intergenic regions of clinical strains cultured *in vitro*. The *cysS-vacA* intergenic regions were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

```
T T T C T C T C G C T T T A G T A G G
                    250
                                                  Strain   vacA titre   vacA type
. . . . . . . T . . . . . . . . . . . .           192      7.52         s1m1i1
. . . . . . . T . . . . . . . . . . . .           173      7.32         s1m1i1
. . . . . . . . . T . . . . . C . . .             206      5.89         s1m1i1
. . . . . . . T . . . . . . . . . . . .           126      4.85         s1m2i1
. . . . . . . . . . . . . . . . . . . .           187      4.84         s1m1i1
. . . . . . . T . . . . . . . . . . . .           201      3.93         s1m1i1
. . . . . . . T . . . . . . . . . . . .           221      3.61         s1m2i2
. . . . . . . T . . . . . . . . . . . .           182      3.01         s1m1i1
. . . . . . . . . . . . . . . . . . . .           249      2.32         s1m2i1
. . . . . . . . . T . . . . . C . . .             122      2.02         s1m1i2
. . . . . . . T . . . . . . . . . . . .           60190    1.92         s1m1i1
. . . . . . . T . . . . . . . . . . . .           124      1.04         s1m1i1
. C . . . . . . . . . . C . . . . G . .           242      0.98         s2m2i2
. . . . . . . T . . . . . . . . . . . .           72       0.87         s1m1i1
. . . . . . . . . . . . . . . . . . . .           83       0.81         s1m1i1
. . . . . . . . . . . . . . . . . . . .           181      0.79         s1m2i2
. . . . . . . . . . . . . . . . . . . .           86       0.58         s1m1i1
. . . . . . . . . . . . . . . . . G . .           Tx30a    0.58         s2m2i2
. . . . . . . T . . . . . . . . . . . .           102      0.46         s1m2i1
. C . . . . . . . . . . . . . . . . G . .         233      0.44         s2m2i2
. . . . . . . . . . . . . . . . . . . .           120      0.41         s1m1i1
. . . . . . . T . . . . . . . . . . . .           46       0.38         s1m1i1
. . G . . . . . . . T . . . . . C . . .           45       0.34         s1m1i1
. . . . . . . . . . . . . . . . . . . .           77       0.26         s1m1i1
. . . . . . . T . . . . . . . . . . . .           73       0.22         s1m2i2
. . . . . . . . . . . . . . . . . G . .           194      0.22         s1m2i1
. . . . . . . . . . . . . . . . . . . .           218      0.15         s1m1i1
. . . . . . . . . T . . . . . C . . .             253      0.14         s1m1i1
. . . . . . . . . T . . . . . C . . .             121      0.08         s1m1i1
. . . . . . . T . . . . . . . . . . . .           93       0.01         s1m2i1
```

Comparison of the *cysS – vacA* intergenic regions of clinical strains cultured *in vitro*. The *cysS-vacA* intergenic regions were aligned using the MegAlign program (DNAstar), and ranked in descending order according to the level of *vacA* mRNA/16s rRNA determined using real-time. The significance of differences in transcript levels between strains with different bases present within the *vacA* promoter were analysed using the Mann-Whitney U test. Nucleotides that match the consensus sequence are represented by a dot. The *vacA* mRNA levels are reported next to the analysed promoter along with the strain name and *vacA* type.

4.3.6 The level of *vacA* mRNA *in vivo* does not correlate with the level of *vacA* mRNA *in vitro*

To determine whether there is a correlation between the level of *vacA* transcription in gastric biopsies and the same clinical strains cultured *in vitro*; *vacA* mRNA levels *in vitro* were plotted against *vacA* levels *in vitro*. The scatter plot shows there is no correlation between *vacA* transcription levels *in vivo* and *in vitro* (Figure 4.7).

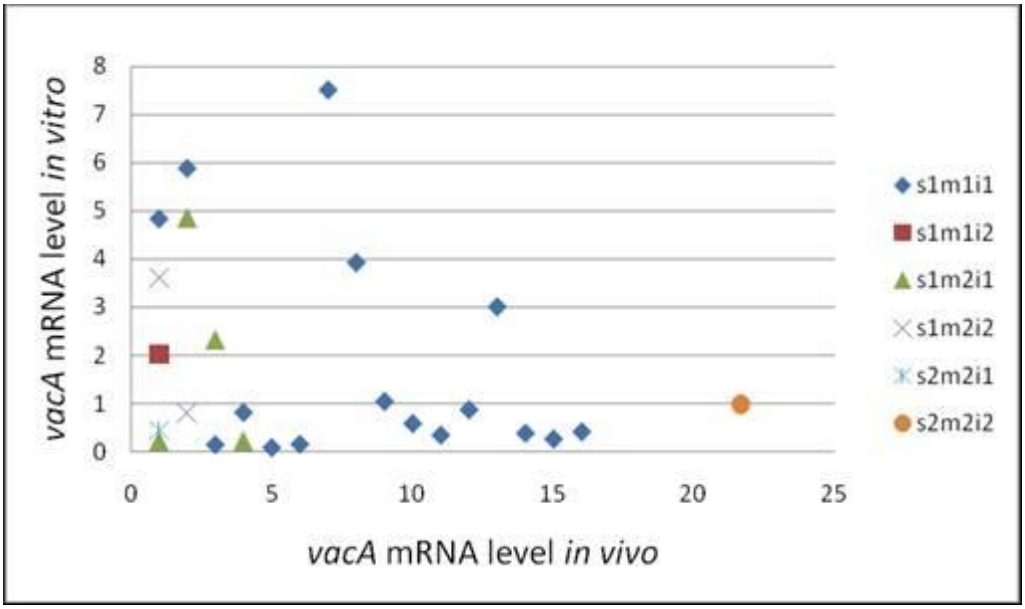


Figure 4.7 *vacA* mRNA level *in vivo* does not correlate with *vacA* mRNA level *in vitro*. The scatter plot above shows the relative level of *vacA* mRNA *in vitro* plotted against the relative level of *vacA* mRNA *in vivo*. The strains were grouped based on their *vacA* type to assess whether there was a type specific correlation.

4.3.7 Transcriptional readthrough from *cysS* is not responsible for the *vacA* mRNA level of a *H. pylori* strain.

To confirm that transcriptional readthrough from the upstream gene *cysS* was not responsible for the differences in *vacA* mRNA levels between strains. PCR using primers A1043 and C1226 was carried out using cDNA synthesised from RNA extracted from broth cultures of 7 clinical strains (conditions detailed in 2.5.4). A1043 binds 9 bp downstream of *cysS* and 101 bp upstream of the *vacA* transcription start site. Genomic DNA isolated from strain 60190 was used as a positive control for the PCR. A product of approximately 479 bp was visible in the control lane containing PCR product amplified from genomic DNA from strain 60190. No bands were visible in lanes containing PCR product amplified from cDNA from clinical strains 60190, 206, 218, 126, 72, 187 and 242 indicating that there was no transcriptional readthrough from *cysS* (Figure 4.8 (A)).

To ensure that the cDNA used in the PCR was of a sufficient concentration an additional PCR was carried out on the same cDNA using primers A3436 and C1226 which bind within the coding sequence of *vacA*. Genomic DNA from strain 60190 was again used as a positive control for the PCR using PCR conditions detailed in section 2.5.4. Bands were visible at approximately 259 bp (for *vacA* type s1) and at 286 bp (for *vacA* type s2) in lanes containing PCR product amplified from cDNA from clinical strains using primers A3436 and

C1226, indicating that the concentration of cDNA used in the PCR was sufficient for amplification (Figure 4.8 (B)).

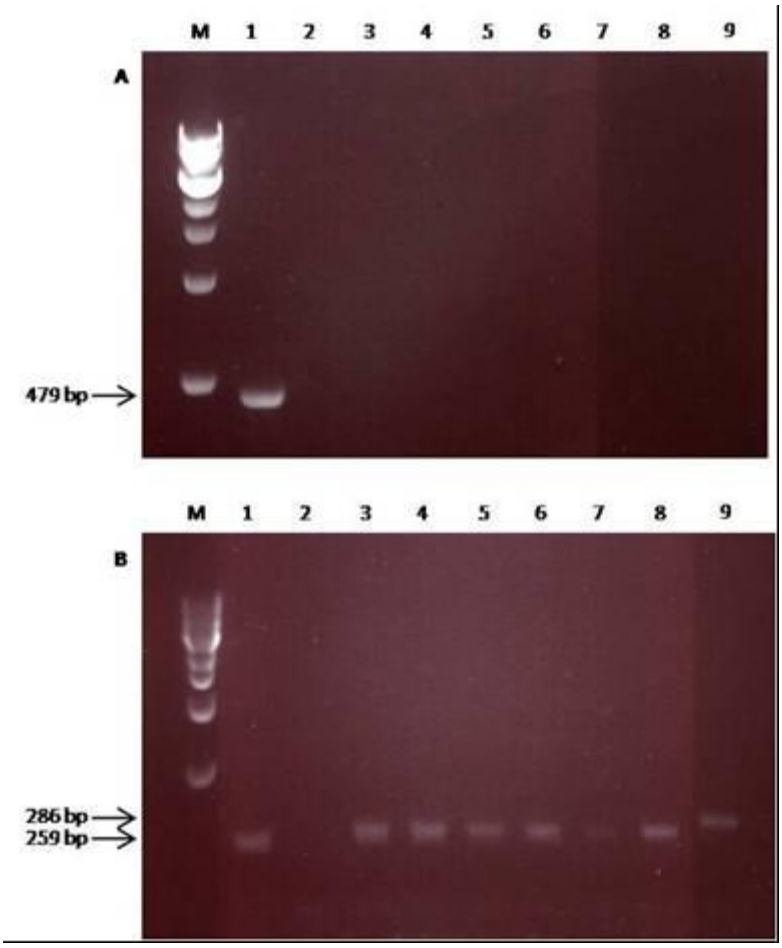


Figure 4.8 *vacA* mRNA levels are not due to transcriptional readthrough from *cysS*. A 2% agarose gel containing PCR products amplified using primers A1043 and C1226. Lane M contains a 1kb DNA ladder, lane 1 contains PCR product amplified from genomic DNA from strain 60190, lane 2 contains a PCR blank, lanes 3 to 9 contain PCR product amplified from cDNA from clinical strains 60190, 206, 218, 126, 72, 187 and 242. Figure 4.8 (B) shows a 2% agarose gel containing PCR products amplified using primers A3436 and C1226 which bind within the coding sequence of *vacA*. Lane M contains a 1kb DNA ladder, lane 1 contains PCR product amplified from genomic DNA from strain 60190, lane 2 contains a PCR blank, lanes 3 to 9 contain PCR product amplified from cDNA from clinical strains 60190 (s1), 206 (s1), 218 (s1), 126 (s1), 72 (s1), 187 (s1) and 242 (s2).

4.4 Discussion

Large variations in *vacA* mRNA levels between strains were observed. 752 fold differences were seen *in vitro*, and relative levels of *vacA* mRNA *in vivo* ranged from 30.22 to undetectable. Several polymorphic differences were identified within the *vacA* promoter, and naturally occurring differences within the -35, -10 and the extended -10 motifs were identified. In agreement with previous studies on the *vacA* promoter *in vitro*, polymorphic differences were seen at position -32 within the -35 motif where there was a frequent G to A substitution (Forsyth *et al*., 1998, Narayanan, 2005). Ayala *et al.*, (2004) also found that the differences in *vacA* expression between 8 strains *in vitro* were unrelated to -10 and -35 sequences. Furthermore promoter exchange experiments between strains 60190 (Tox+) and 86-313 (Tox-) show that although the two strains show differences in *vacA* mRNA levels, the differences must be dictated by sequences outside the -10 and -35 motifs as the two regions are identical in the two strains (Forsyth and Cover, 1999).

Forsyth *et al.*, (1999) carried out site directed mutagenesis on the *cysS* – *vacA* intergenic region to determine the importance of the promoter sequences on *vacA* transcription using a *xylE* reporter system. Site directed mutagenesis of the -10 region where the wild type sequence TAAAAG was changed to AGATCT resulted in a 15 fold reduction in *vacA* level. Changing the -35 motif from the wild type sequence TTTATG to AGATCT resulted in a 4 fold reduction in expression and the deletion of 6 nucleotides (TTTATG) from position -37 to -32 in strain 60190 resulted in a 6 fold reduction in *vacA* expression (Forsyth *et al.*, 1998).

Narayanan (2005) analysed the *vacA* promoter sequences of a collection of published strains and found that in 4 out of 8 clinical strains there was a guanine to adenosine substitution at position -14. However, in the study by Forsyth *et al.,* 1999 substitution of guanine to a thymine at the -14 position using site directed mutagenesis resulted in a 2 fold reduction in *vacA* transcript level (Forsyth *et al.,* 1999). Substitutions at the -13 and -15 positions had no effect on *vacA* transcript levels (Forsyth *et al.,* 1998). This lead to the conclusion that positions -12 and -14 may be important for RNA polymerase binding (Forsyth *et al.,* 1998). In this study a guanine to adenosine substitution was also found at position -14 within the extended -10 motif. However differences in the *vacA* transcript level between the different strains *in vivo* and *in vitro* were not significantly associated with the polymorphic differences present at these sites.

In this study, polymorphic differences were observed in the spacer region between the -10 and the -35 motifs, however polymorphic differences in this region were not associated with differences in *vacA* mRNA levels between strains. The finding that the specific sequence of the spacer region was not associated with differences in *vacA* mRNA level in this study agrees with the findings of Forsyth *et al.,* 1999 where substituting CCTTA to GATCT from positions -23 to -19 did not affect levels of *vacA* transcription. However Forsyth *et al* concluded that spacing appeared to be important as introducing a 10bp insertion between -24 and -23 reduced expression. Spacing of the -10 and -35 motifs in the clinical strains investigated in this study was well conserved

(apart from one strain in which a thymine residue was inserted at position -19 (Figure 4.1 and Figure 4.6)).

Prokaryotic mRNAs can have a half life between just a few minutes to several hours (Brawerman, 1987). Variation in mRNA stability in prokaryotes suggests that its rate of decay is involved in the regulation of gene expression. The complex mechanisms that determine mRNA stability are beginning to be understood (reviewed in Regnier and Arriano, 2000). *H. pylori* encodes at least four genes involved in RNA processing and degradation which include *papS*, *rnc*, *rnpA* and *pnp* (Tomb *et al*., 1997). Products of these genes are likely to form part of the degradosome in *H. pylori*. Therefore it would be likely that *H. pylori* utilises the differences in mRNA stability to regulate gene expression. Furthermore, *H. pylori* has been shown to utilise mRNA decay to regulate expression of the urease gene cluster in response to acidic pH (Akada *et al*., 2000).

A stem loop structure beginning at the +4 site of the mRNA transcript has been identified previously by analysing the upstream region of sequenced strains using such secondary structure programs as GeneQuest (DNAstar Inc) (Narayanan, 2005). The stem loop structure consists of an 11 bp stem that is strengthened by strong GC base pairing at the base of the stem and a loop which is 5 nucleotides in length. This stem loop structure is well conserved. However, in this study, strains possessing an A at position +28 within the second stem of the stem loop were found to express more *vacA* than those that have a G at this position (P = 0.0089).

Previous studies have shown that 5' terminal secondary structures are essential for the stability of some mRNAs (Bechhofer and Dubnau, 1987, Belasco *et al*., 1986). Emory *et al.,* (1992) identified a stem loop in the 5'UTR of *E. coli ompA,* which was essential for its function as an mRNA stabiliser. This 5' stem loop protects the *ompA* transcript from degradation by RNaseE, that appears to begin downstream of the 5' terminus. Deletion of this stem loop resulted in reduced stability of the *vacA* transcript, although no effect was seen on VacA protein levels in a stem loop deletion mutant (Narayanan, 2005). In contrast to this finding, promoter exchange experiments were conducted by Forsyth *et al.,* 1999, in which the entire 5' untranslated region of *vacA* from strain 86-313 (Tox-) was replaced with that of strain 60190 (Tox+) no increase in *vacA* mRNA level was observed in the chimeric strain, suggesting that sequences in the 5' untranslated region do not significantly affect *vacA* mRNA stability. However the untranslated regions were not sequenced in the 2 strains and it may be that the untranslated regions do not differ between the two strains investigated in the study. Ayala *et al*., (2004) also compared the 5' untranslated mRNA regions of 8 *H. pylori* strains. They found that differences in *vacA* transcription were unrelated to sequence differences in this region. However, their analysis did not include the putative 5' stem loop that starts at position +4 and ends at position +30 as only sequences between the position +33 and the ATG start site were assessed.

The presence of varying numbers of binding sites for transcription factors could potentially explain the differences in *vacA* mRNA level between strains.

Inverted repeats often represent binding sites for transcriptional regulators. Ayala *et al.*, identified an inverted repeat upstream of the -35 motif in *vacA* that was present in 4 out of 8 strains. However its role in determining transcript levels was not determined. In this study it was observed that strains possessing a perfect inverted repeat expressed significantly higher levels of *vacA in vitro*. However deleting a 27 bp region from -66 to -40, as well as a 42 bp region from -108 to -67, resulted in no change in *vacA* transcript levels in strain 60190 VX-1 CAT when compared to the control (Forsyth and Cover, 1999).

No correlation was found between *vacA* transcription levels *in vivo* and *in vitro* using matched strains and biopsies. Regions within the promoter associated with differences in *vacA* production between strains also differed *in vitro* and *in vivo*. Different and opposing effects *in vitro* and *in vivo* may imply that *vacA* is regulated *in vivo*, and could be regulated in response to growth phase of the bacterium or by environmental factors. The majority of bacteria are thought to be in a state of non-growth in their natural environment, persisting in a nutrient-limited stationary phase (Kjelleberg *et al*., 1993). Difference in growth phase may explain the difference in *vacA* transcript levels between the same strains *in vivo* and *in vitro*. This is supported by several studies including microarray analysis which demonstrated that *vacA* expression is repressed after acid exposure at pH 5.0 (Merrell *et al*., 2003a). This was in contrast to non-array based work which demonstrated that *vacA* was induced under acidic conditions (Barnard *et al*., 2004). Furthermore, RNA dot blot analysis revealed a significant increase in the accumulation of *vacA* transcripts under iron-restricted conditions (Szczebara *et al*., 1999). Other studies have investigated

the affect of adhesion to host cells on *vacA* transcription, (Gieseler *et al*., 2005) observed using real-time, quantitative PCR (RT-qPCR) that expression of *vacA* increased during infection of AGS cells for 4 out of 8 *H. pylori* strains studied. Similar results were also obtained during infection of polymorphonuclear leukocytes in which 2 out of 8 strains produced higher levels of *vacA* mRNA. This observation has been confirmed by the previous work of van Amsterdam *et al.*, (2003) in which it was observed that *vacA* expression was induced upon attachment to the human gastric cell line HM02. Furthermore, the level of *vacA* was significantly reduced in coccoid forms of *H. pylori* which are supposed to represent a viable but non-culturable form of *H. pylori* (Monstein and Jonasson, 2001).

4.4.1 Conclusions

Differences in sequences within the -35 and extended -10 motifs were not found to be significantly associated with differences in levels of *vacA* transcription *in vivo* or *in vitro*. However sequence variations at other positions within the sequence may have opposing effects. No correlation was found between *vacA* transcription levels *in vivo* and *in vitro* which may imply that *vacA* is regulated *in vivo*.

Polymorphic differences at position +28 located within a putative stem loop in the 5' UTR of *vacA* were significantly associated with *vacA* mRNA levels *in vivo*. Stem loops at this position are often involved in mRNA stability and may

provide a mechanism for determining *vacA* mRNA levels between different *H. pylori* strains although this would have to be investigated further.

It is possible that differences in *vacA* transcript levels between strains may be due to the effect of a transcriptional activator or repressor. Inverted repeats are often binding sites for transcriptional regulators. An inverted repeat located upstream of the -35 region in the *vacA* promoter has been identified previously. In this study it was observed that strains possessing a perfect inverted repeat expressed significantly higher levels of *vacA in vitro*. Mutagenesis work would have to be carried out to determine the effect of these positions on *vacA* expression. Further study of these regions may provide an insight into the process of transcriptional regulation in *H. pylori* and may be relevant to understanding how the bacterium persists in its gastric niche.

4.5 Future Work

In order to determine whether naturally occurring sequence differences between strains within the upstream inverted repeat and the 5' UTR stem loop are relevant in determining *vacA* transcript levels. Site directed mutagenesis would have to be carried out on the regions of interest. This will be investigated in chapter 5.

In addition, differences in the VacA signal sequences may be involved in the efficiency of VacA secretion and may account for the heterogeneity in VacA protein levels between strains. The concentration of VacA in broth culture

supernatants were 10-fold higher in Tox+ strain 60190 than in Tox- strain 86-338 even though *vacA* transcript levels did not differ significantly between the two strains. The effect of signal sequence differences on secreted VacA protein levels will be investigated further in chapter 6.

Chapter 5

5 Investigation into the effect of an upstream inverted repeat and a 5' stem-loop structure in the *vacA* promoter region on *vacA* mRNA levels.

5.1 Introduction

In Chapter 4 the effect of naturally occurring differences in the *cysS – vacA* intergenic region on *vacA* transcript levels *in vivo* and *in vitro* were investigated. A region of particular interest *in vivo* was a stem-loop structure at the +4 site of the *vacA* 5' untranslated region (UTR), identified previously by Narayanan (2005). Work in chapter 4 showed that this region is polymorphic, particularly at position +28. In 11 out of 27 strains an A was present at this position whereas in the other 16 strains a G was present at this position. Interestingly, strains with an A at this position had significantly higher levels of *vacA* mRNA than those with a G at this position (*P*= 0.0062). This region was associated with high *vacA* transcript levels *in vivo* but not *in vitro*.

The potential to form a 5' stem-loop is a common feature in many stable prokaryotic mRNAs, and such stem-loop structures have been implicated in mRNA stabilisation of many prokaryotic transcripts (Emory *et al*., 1992, Belasco *et al*., 1986, Hambraeus *et al*., 2000, Diwa *et al*., 2000, Xia *et al*., 2002, Bricker and Belasco, 1999, Unniraman *et al*., 2002a, Unniraman *et al*., 2002b, Bechhofer and Dubnau, 1987, Sandler and Weisblum, 1989, Sandler and Weisblum, 1988). 5' stem-loop elements protect mRNA from degradation by the degradative endoribonuclease RNase E by inhibiting its binding to the free 5' end the mRNA (Belasco *et al*., 1986, Bouvet and Belasco, 1992,

Ehretsmann *et al*., 1992a, Emory *et al*., 1992, Hansen *et al*., 1994). Previous studies have found that the length of the stem structure, the size of the loop, the folding energy of the stem-loop and the length of the single stranded region prior to the stem-loop were all factors of 5' stem-loops that affect mRNA stability (Bechhofer and Dubnau, 1987).

Another endoribonuclease, RNase III, can initiate mRNA degradation by removing stabilizing 5' stem-loop structures (Matsunaga *et al*., 1996a, Matsunaga *et al*., 1996b), allowing the downstream mRNA to decay at a rate determined by nucleases other than RNase III. RNase III cleaves double stranded RNAs of low sequence complexity. Recognition of the substrate by RNase III is dependent on the length of the double helical structure with a minimum length of one full turn, or approximately 11bp (Court *et al*., 1993, Nicholson *et al*., 1999, Robertson *et al*., 1982). Two specific regions, a 3-bp proximal box (pb) and a 2-bp distal box (db) are also involved in RNase III binding (Zhang and Nicholson, 1997). The presence of certain Watson-Crick base pairs at specific positions within the proximal and distal boxes strongly inhibit cleavage by RNase III (Zhang and Nicholson, 1997).

mRNA degradation has been shown to be an important mechanism for controlling gene expression by controlling the availability of transcripts for translation (Emory *et al*., 1992, Hambraeus *et al*., 2000). The ability of some bacteria to selectively degrade mRNAs allows them to alter their patterns of gene expression in response to changes in their environment.

The 5’ stem-loop in *vacA* appears to be well conserved among clinical isolates and is present in all clinical *H. pylori* strains investigated and begins at the +4 site. It consists of an 11bp stem that is strengthened by strong GC base pairing at the base of the stem and contains a loop which is 5 nucleotides in length. However, 11 out of 27 strains studied in chapter 4 possess an A at position +28 whereas the other 16 strains had a G at this position (Figure 5.1.). Deletion of the 5’ stem-loop in a previous study showed *vacA* transcripts were less stable in stem-loop deletion mutant than transcript from the wild-type parent strain (Narayanan, 2005). Natural polymorphic differences in the 5’ stem-loop between strains appear to be associated with *vacA* mRNA level *in vivo* and could potentially contribute to *vacA* heterogeneity *in vivo*.

The relative stability of the *vacA* transcripts in the parent strain 60190/pCTB2::*cat* and the stem-loop deletion mutant 60190/ΔSL were investigated by Narayanan, 2005 by treating broth cultures of each of the two strains with rifampicin, which inhibits RNA polymerase by binding in the RNA exit channel which leads to a halt in transcription. By Northern blotting it was found that detectable *vacA* transcript from the parental strain 60190/pCTB2::*cat* gradually decreased over two hours after the addition of rifampicin, whereas the stem-loop deletion mutant 60190/ΔSL contained no detectable *vacA* transcript 5 minutes after the addition of rifampicin, indicating that the *vacA* transcript produced by mutant strain 60190/ΔSL was less stable than that of strain 60190/pCTB2::*cat*. Surprisingly when VacA protein levels were assessed in 24 hour broth cultures no significant difference in VacA levels between the mutant and the parent strain was observed, possibly

indicating that there was a problem with the methodology or that deletion of the stem-loop did not affect the steady-state levels of *vacA* mRNA when grown for 24 hours (Narayanan, 2005). A reduction in *vacA* stability was seen only in the rifampicin experiment. Cultures used for protein analysis, were not treated with rifampicin, which may explain the disparity between the mRNA and protein data.

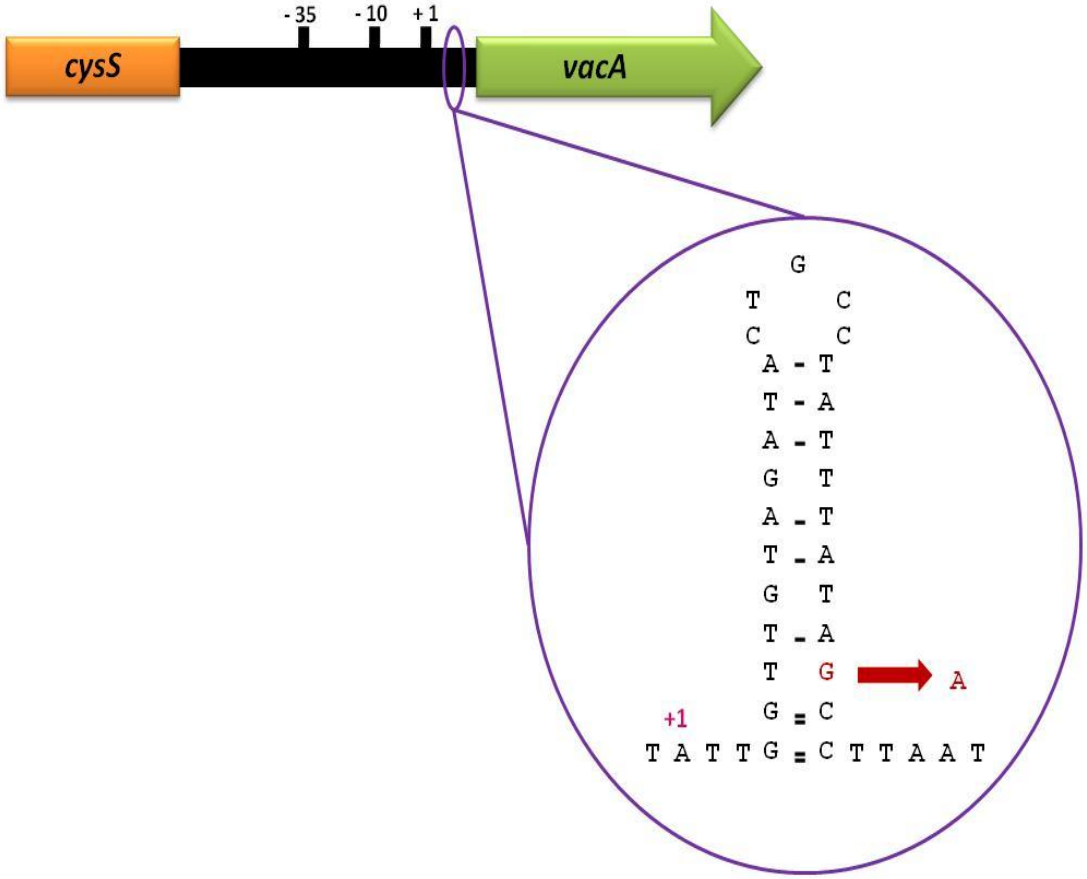


Figure 5.1 The putative stem-loop begins at the +4 site of the 5' untranslated region of *vacA*. A Guanine to Adenine substitution is present at position +28 in 11 out of 27 strains tested in chapter 4 (Adapted from Narayanan, 2005).

Another potential region of interest highlighted in chapter 4 was the inverted repeat upstream of the -35 motif identified by Ayala *et al.*, (2004). Sequence alignment reveals that strains with a fully palindromic inverted repeat express significantly more *vacA* than other strains (P= 0.01).

A large number of perfect inverted repeats have been identified in prokaryotic genomes. These inverted repeats may represent transcription factor binding sites (Ladoukakis and Eyre-Walker, 2008). The presence of, and varying numbers of transcription factor binding sites with differences in binding affinity between strains could contribute to differences in *vacA* transcript levels observed between clinical isolates seen in chapter 4.

Inverted repeats have also been shown to act as sites for genetic rearrangements mediated by misalignment and can cause duplications or deletions which include the repeat itself and any intervening sequence. Perfect inverted repeats can arise when one copy of a small, imperfect, inverted repeat corrects another copy of the inverted repeat via strand misalignment (Lovett, 2004).

5.1.1 Aims

The aims of this chapter were to investigate the role of the naturally occurring polymorphic differences in the 5' UTR stem-loop and the inverted repeat upstream of the -35 motif and their role in determining *vacA* transcript levels. Site directed mutagenesis and real-time PCR were used to compare the *vacA*

transcript levels of the mutant and wild-type parent strains. The presence of potential regulator binding sites in the region upstream of the -35 motif was also investigated using a computational prediction program.

5.2 Results

5.2.1 Characterisation of the effect of deletion of the 5' stem-loop on *vacA* mRNA levels

To confirm the results observed by G.L. Narayanan (described in section 5.1), and to begin to optimise the culture conditions to characterise the mutant strain constructed in this study, genomic DNA was isolated from strain 60190/ΔSL (prepared previously by Narayanan (2005) - details of the production of this mutant are outlined in Figure 5.2.). A 578bp region including 120 bp upstream of the *vacA* start codon, the region encoding the VacA signal sequence and the first 252 base pairs of the region encoding the mature cytotoxin was amplified by PCR using primers A1043 and C1226. The PCR products were purified and sequenced from primer A1043 using an Applied Biosystems 3100 Genetic analyser (Biopolymer Synthesis and Analysis unit, University of Nottingham, UK). Sequences retrieved were aligned to 60190/pCTB2::*cat* using the MegAlign program (DNAstar). Sequencing confirmed the presence of the expected mutation (Figure 5.2) and that no secondary mutations were present.

Broth cultures of strains 60190/pCTB2::*cat* and 60190/ΔSL were prepared as described in section 2.2.5 and grown with shaking at 100 rpm for 18 hours. Three separate cultures were prepared on separate occasions. Broth culture

supernatants were recovered by centrifugation (as detailed in section 2.7.1) and VacA protein levels assessed by Western blotting using the polyclonal primary antibody anti-VacA p58 using the methodology detailed in section 2.7.5, and density of the bands was determined using densitometry as described in section 2.7.6. Samples were also taken from the same broth cultures for RNA extraction at 18 hours and the *vacA* mRNA level per bacterial cell was assessed by real-time PCR using the methodology described in section 4.3.3. Quantitative real-time PCR was performed in triplicate for each broth culture tested revealed that there were no significant differences in *vacA* transcript levels between the mutant strain 60190/ΔSL and the control strain 60190/pCTB2::*cat* when strains were grown in liquid culture for 18 hours with mean *vacA* mRNA levels of 0.07 and 0.073 respectively (Figure 5.3). Western blot analysis showed that there were no significant differences in VacA protein levels between strain 60190/ΔSL and 60190/pCTB2::*cat* with mean relative densitometry values of 0.62 and 0.63 respectively (Figure 5.4).

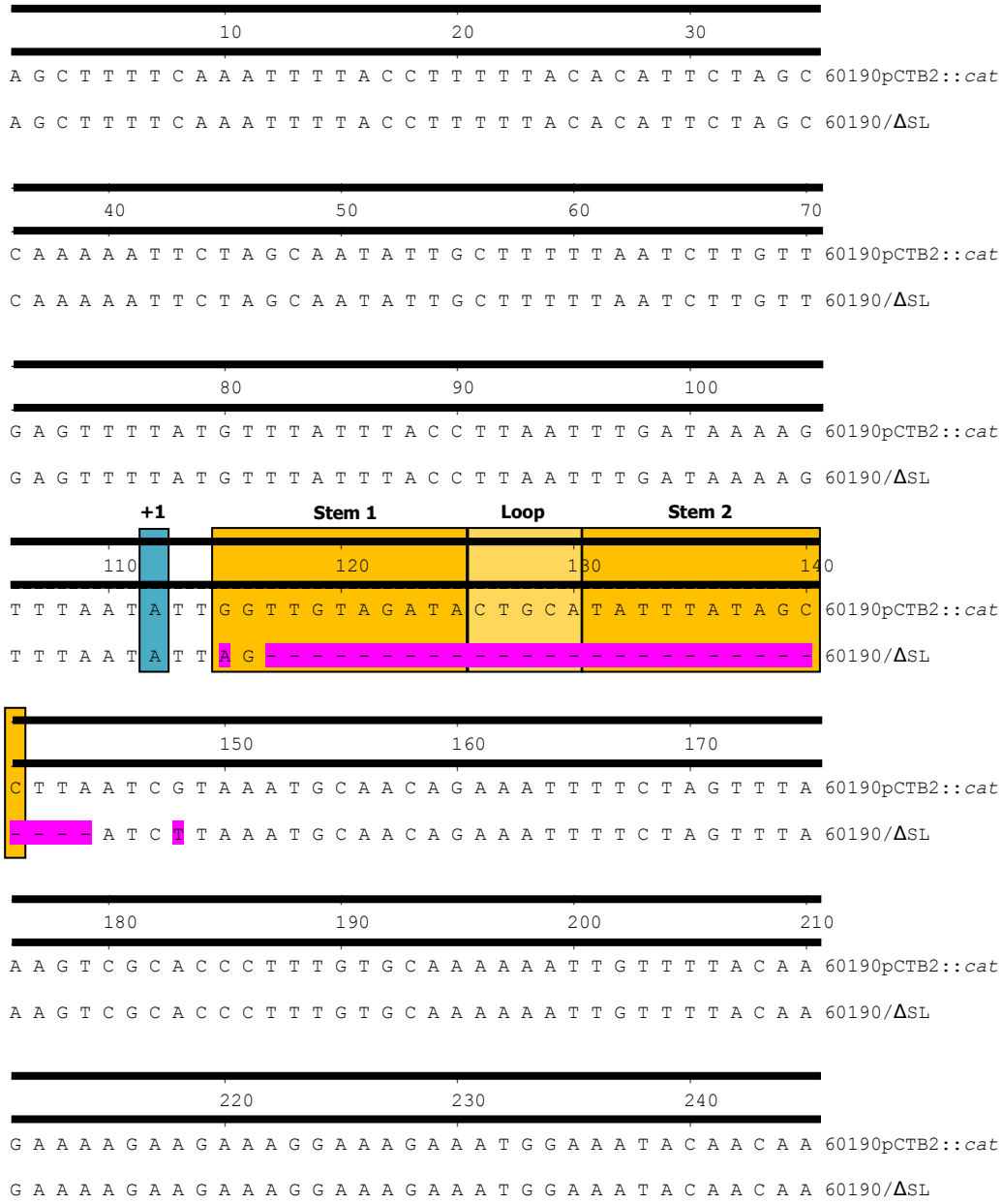


Figure 5.2 Sequence Alignment of 60190/ΔSL. The stem-loop deletion mutant 60190/ΔSL and the control strain 60190/pCTB2::cat were sequenced using primer A1043. Sequences were aligned using the MegAlign program (DNAStar). The stem-loop region is highlighted in orange and the +1 site in blue. Mutated nucleotides are highlighted in pink. Mutant 60190/ΔSL was constructed previously by G.L Narayanan using inverse PCR using Pfu polymerase and pCTB2::cat as a template. Primers were designed to flank the stem-loop region resulting in deletion of the stem-loop. Bgl II sites were added to the 5' ends of the primers. PCR products were purified and digested with Bgl II followed by Dpn I treatment to remove methylated template DNA. The Bgl II sites were ligated with T4 ligase then transformed into *E. coli* Novablue cells. Antibiotic resistant colonies were selected; the DNA isolated then sequenced using primer C1226. The ligation reactions were transformed by homologous recombination into *H. pylori* strain 60190. Genomic DNA was extracted and PCR amplified using primer sets DL1, C0374 to confirm the correct location of the insertion and primers A1043, C1226 to confirm the presence of the correct mutation and the absence of secondary mutations.

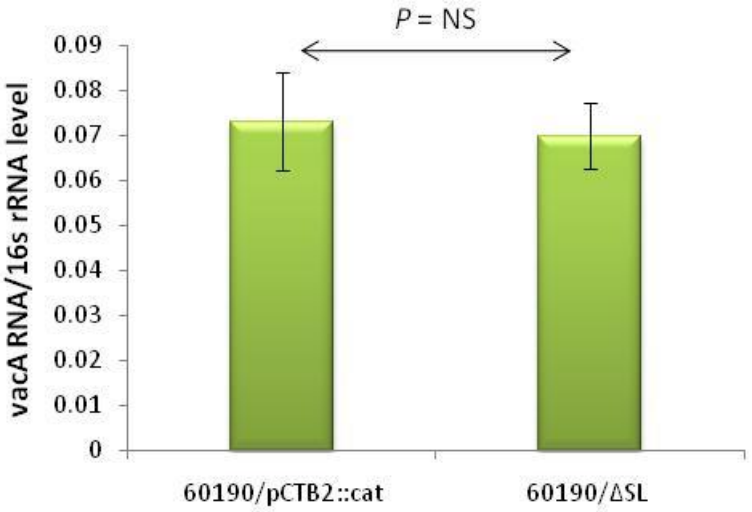


Figure 5.3 *vacA* mRNA levels of strains 60190/pCTB2::*cat* and 60190/ΔSL grown for 18 hours in broth culture. Strains 60190/ΔSL (n=3) and control strain 60190/pCTB2::cat (n=3) were grown in broth culture for 18 hours and *vacA* mRNA levels were determined using real-time PCR. Mean *vacA* mRNA levels of 0.07 and 0.073 for 60190/ΔSL and 60190/pCTB2::*cat* respectively. The difference in the means of two strains was determined by t-test: two sample assuming unequal variance (two-tail). Error bars show the standard error.

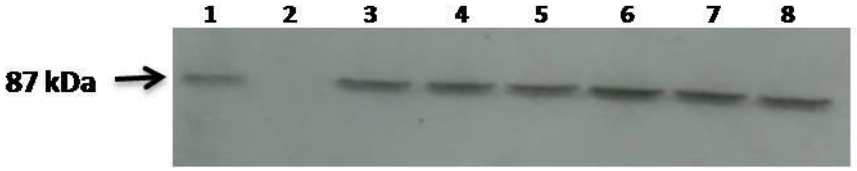


Figure 5.4 VacA Western blot of control strain 60190/pCTB2::*cat* and stem-loop deletion mutant 60190/ΔSL. 18 hour broth culture supernatants were resolved by SDS-PAGE and immunoblotted with anti-VacA p58 antibody. Broth cultures were corrected to the same cell density and equal sample volumes loaded as follows: strain 60190 (lane 1), A130 (lane 2), 60190/pCTB2::*cat* culture 1, 2 and 3 (lanes 3, 4 and 5 respectively), 60190/ΔSL culture 1, 2 and 3 (lanes 6, 7 and 8 respectively).

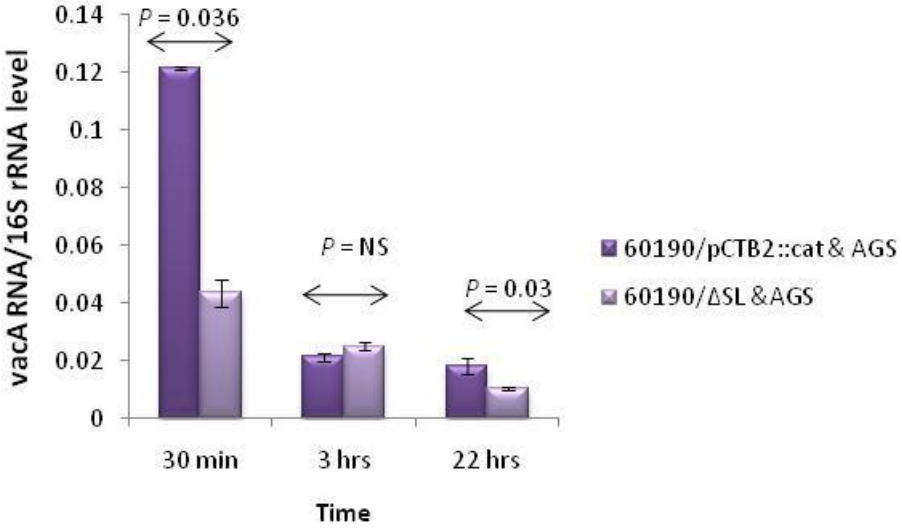
5.2.2 Effect of co-culture on *vacA* mRNA levels

Emory *et al* 1992 reported that the stabilising effect of a 5' stem-loop structure in *E. coli ompA* was greater under conditions of metabolic stress. Additionally the presence of adenosine rather than guanine at position +28 of the *vacA* 5' UTR was associated with high *vacA* transcript levels *in vivo* but not *in vitro*. To begin to try to recreate *in vivo* culture conditions the mutant strain 60190/ΔSL and control strain 60190/pCTB2::*cat* were co-cultured with AGS cells using the methodology outlined in section 2.2.7 As a negative control wells containing *H. pylori* strains 60190/pCTB2::*cat* and 60190/ΔSL and no AGS cells were prepared in the same way and included on the same 24 well plate . The number of cultures analysed for each strain from each time point for each growth condition used are detailed in Figure 5.5 (A) and (B). Culture samples were removed from the wells by manual agitation after 30 minutes, 3 hours and 22 hours of incubation and *vacA* transcript levels assessed using quantitative real-time PCR as described in section 4.3.3.

When co-cultured with AGS cells, a 67% and 42% reduction in *vacA* transcript levels were observed in strain 60190/ΔSL at 30 minutes and 22 hours respectively when compared to that of the control strain 60190/pCTB2::*cat*. No significant difference in *vacA* mRNA levels was seen at 3 hrs (Figure 5.5 (A)). *vacA* mRNA levels were reduced by 62% and 72% in strain 60190/ΔSL compared to strain 60190/pCTB2::*cat* at 30 minutes and 22 hours when *H. pylori* cells were cultured in F12 Ham medium without AGS cells (Figure 5.5 (B)). Again, no significant reduction in *vacA* transcript levels was observed in

strain 60190/ΔSL when cultured in F12 Ham medium for 3 hours. *vacA* mRNA levels in both strains (60190/pCTB2::*cat* and 60190/ΔSL) were approximately 70% higher after 30 minutes when strains were co-cultured with AGS cells. *vacA* mRNA levels in strain 60190/pCTB2::*cat* were 50% higher at 22hrs when cultured in F12 Ham medium without AGS cells. No significant difference in *vacA* transcript levels in strain 60190/ΔSL was observed at 22hrs when the two growth conditions were compared. No significant differences in *vacA* transcript levels were observed in either strain when the two growth conditions were compared at 3 hours (Figure 5.5 (A) and (B)).

A



B

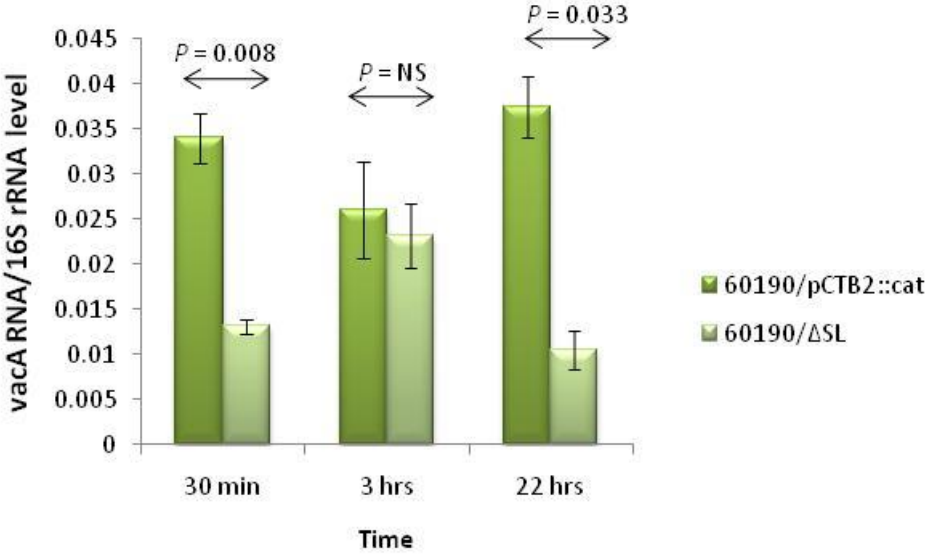


Figure 5.5 *vacA* mRNA levels of strains 60190/pCTB2::cat and 60190/ΔSL grown for 30 minutes, 3 hours and 22 hours in (A) co-culture with AGS cell and (B) in F12 Ham medium without AGS cells determined using quantitative real-time PCR. (A) Mean *vacA* mRNA levels when co-cultured with AGS cells 60190/pCTB2::cat 30 minutes 0.12 (n=3), 3 hours 0.021 (n=6), 22 hours 0.018 (n=6); 60190/ΔSL 30 minutes 0.043 (n=2), 3 hours 0.025 (n=6), 22 hours 0.01 (n=6). (B) Mean *vacA* mRNA levels when cultured in F12 Ham medium 60190/pCTB2::cat 30 minutes 0.034 (n=3), 3 hours 0.026 (n=2), 22 hours 0.037 (n=2); 60190/ΔSL 30 minutes 0.013 (n=3), 3 hours 0.023 (n=2), 22 hours 0.01 (n=2). The difference between the means of two strains was determined by t-test: two sample assuming unequal variance (two-tail). Error bars show the standard error.

5.2.3 *H. pylori* culture in F12 Ham medium was optimised for analysis of *vacA* transcript levels at early time points

H. pylori strains 60190/pCTB2::*cat* and 60190/ΔSL were cultured in 10ml of F12 Ham medium supplemented with L-glutamine and 10% FBS as detailed in section 2.2.6 and incubated for either 20 minutes, 40 minutes or 3 hours. Relative *vacA* transcript levels were determined using quantitative real-time PCR as detailed in sections 4.3.3. The number of cultures analysed for each strain from each time point used are detailed in Figure 5.6.

vacA mRNA levels were significantly lower in the stem-loop deletion mutant 60190/ΔSL by 55% (P = 0.008), 46% (P = 0.03) and 66% (P = 0.0024) at time points 20 minutes, 40 minutes and 180 minutes respectively when compared to strain 60190/pCTB2::*cat* (Figure 5.6).

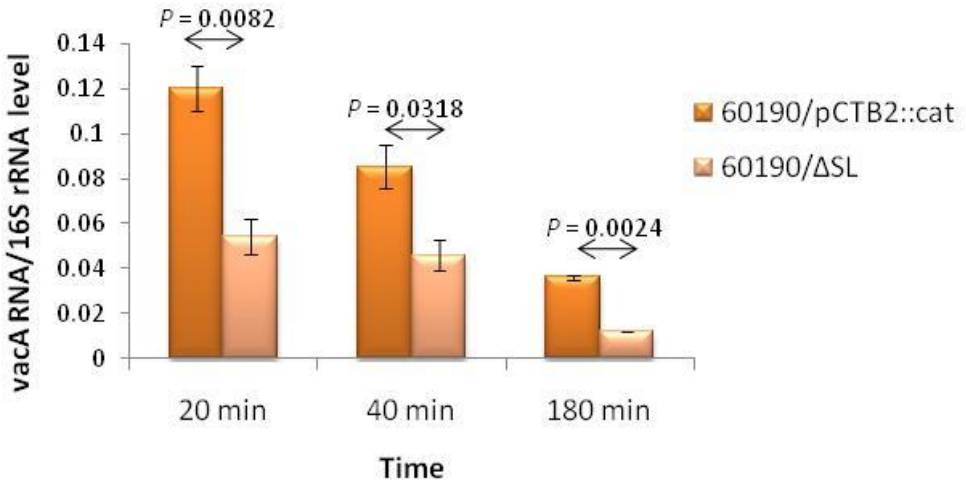


Figure 5.6 *vacA* mRNA levels of strains 60190/pCTB2::cat and 60190/ΔSL grown for 20 minutes, 40 minutes and 180 minutes in 10ml F12 Ham medium determined using quantitative real-time PCR. Mean *vacA* mRNA levels 60190/pCTB2::cat 20 minutes 0.12 (n=3), 30 minutes 0.085 (n=3), 180 minutes 0.036 (n=3); 60190/ΔSL 20 min 0.054 (n=3), 40 minutes 0.046 (n=3), 180 minutes 0.012 (n=3). The difference between the means of two strains was determined by t-test: two sample assuming unequal variance (two-tail). Error bars show the standard error.

5.2.4 Construction of the 5' UTR stem-loop mutant: 60190/SL GtoA

To determine whether the presence of an adenine rather than a guanine at position +28 within the 5' stem-loop of the start of the untranslated region of *vacA* was important in determining differences in *vacA* levels between strains, a guanine to adenine mutation was introduced at position +28 of the *vacA* 5' UTR using the QuikChange site-directed mutagenesis kit (Stratagene, UK). The plasmid pCTB2::*cat* (Forsyth *et al*., 1998) was used as a template which contains the 3' end of *cysS*, the *cysS*-*vacA* intergenic region, 274 bp of *vacA* coding sequence from *H. pylori* 60190 and a chloramphenicol acetyl transferase (*cat*) gene inserted at the 3' terminus of *cysS* (Letley *et al*., 2003). Complementary primers +4 GtoA Forward and +4 GtoA Reverse, 31 nucleotides in length were designed to span the site that was to be mutated with the mutations included in the middle of the primer. These primers were used to replicate the wild type plasmid template pCTB2::*cat* which incorporated the mutation into the resulting plasmids. Mutant plasmids were treated with *Dpn1* to remove the methylated parent plasmid and transformed into NovaXG Zappers™ Electrocompetent cells by electroporation using the manufacturer's protocol. Chloramphenicol resistant colonies were selected and plasmid DNA was extracted and the presence of the mutation confirmed by sequencing, then introduced into *H. pylori* strain 60190 by natural transformation. Mutant *vacA* alleles were incorporated into the chromosomal *vacA* gene by double homologous recombination which was selected for by chloramphenicol resistance. Two independent isolates of colonies of 60190/SL GtoA mutants were selected. To confirm that the insert had transformed into the correct

location in the genome, DNA was isolated from both isolates of 60190/SL GtoA and amplified using primers DL1 and C0374 that bind externally to the inserted region. PCR products amplified from strain 60190/SL GtoA had a product size of approximately 2 Kb, whereas those amplified from the wild-type were approximately 900bp, indicating that the insertion had transformed into the correct location. To confirm the presence of the correct mutation and the absence of secondary mutations 120 bp region upstream of the *vacA* start codon, the region encoding the first 252bp of the region encoding the mature cytotoxin was PCR amplified from genomic DNA of the inverted repeat mutant 60190/SL GtoA using the forward primer A1043 and the reverse primer C1226 then sequenced using primer A1043 using an Applied Biosystems 3100 Genetic analyser (Biopolymer Synthesis and Analysis unit, University of Nottingham, UK). Sequences retrieved were aligned using the MegAlign program (DNAstar). Sequencing confirmed the presence of the expected mutation (Figure 5.7) and that no secondary mutations were present in the *vacA* sequence and the sequence upstream of *vacA.*

5.2.5 Analysis of *vacA* mRNA levels in wild-type and mutant strains

H. pylori strains 60190/pCTB2::*cat*, 60190/ΔSL and 60190/SL GtoA were cultured in 10ml of F12 Ham medium with L-glutamine and 10% FBS, and incubated for 30 minutes and 180 minutes then analysed by real-time PCR using the methodology described in section 4.3.3. Four separate cultures were analysed for each strain at each time point. Two independent isolates of strain

60190/SL GtoA cultured in triplicate and the data from each combined to give a total of eight cultures of strain 60190/SL GtoA in total.

Steady state levels of *vacA* mRNA were significantly higher in strain 60190/SL GtoA at 30 minutes and 180 minutes, with a 2 fold (P = 0.004), and 4.4 fold (P = 0.00028) increase in *vacA* transcript levels respectively when compared to control strain 60190/pCTB2::*cat*. *vacA* mRNA levels were significantly reduced in the stem-loop deletion mutant 60190/ΔSL by 75% (P = 0.0081) and 61% (P = 0.0143) at 30 minutes and 180 minutes respectively when compared to strain 60190/pCTB2::*cat* (Figure 5.8).

```
G C T T T T C A A A T T T T A C C T T T T T A C A C A T T C  Majority
                  10                  20                  30
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/SL GtoA Sc2
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/SL GtoA Sc3

T A G C C A A A A A T T C T A G C A A T A T T G C T T T T T  Majority
                  40                  50                  60
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/SL GtoA Sc2
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/SL GtoA Sc3

A A T C T T G T T G A G T T T T A T G T T T A T T T A C C T  Majority
                  70                  80                  90
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/SL GtoA Sc2
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/SL GtoA Sc3

                                          +1      Stem 1
T A A T T T G A T A A A A G T T T A A T A T T G G T T G T A  Majority
                  100                 110                 120
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/SL GtoA Sc2
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/SL GtoA Sc3

          Loop          Stem 2
G A T A C T G C A T A T T T A T A G C C T T A A T C G T A A  Majority
                  130                 140                 150
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . A . . . . . . . . . . .  60190/SL GtoA Sc2
. . . . . . . . . . . . . . . . . . A . . . . . . . . . . .  60190/SL GtoA Sc3

A T G C A A C A G A A A T T T T C T A G T T T A A A G T C G  Majority
                  160                 170                 180
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/SL GtoA Sc2
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/SL GtoA Sc3

C A C C C T T T G T G C A A A A A A T T G T T T T A C A A G  Majority
                  190                 200                 210
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/SL GtoA Sc2
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/SL GtoA Sc3
```

Figure 5.7 The stem-loop deletion mutant 60190/SL GtoA and the control strain 60190 pCTB2::cat were sequenced using primer A1043. Sequences were aligned using the MegAlign program (DNAStar). The stem-loop region is highlighted in orange and the +1 site in blue. Mutated nucleotides are highlighted in pink.

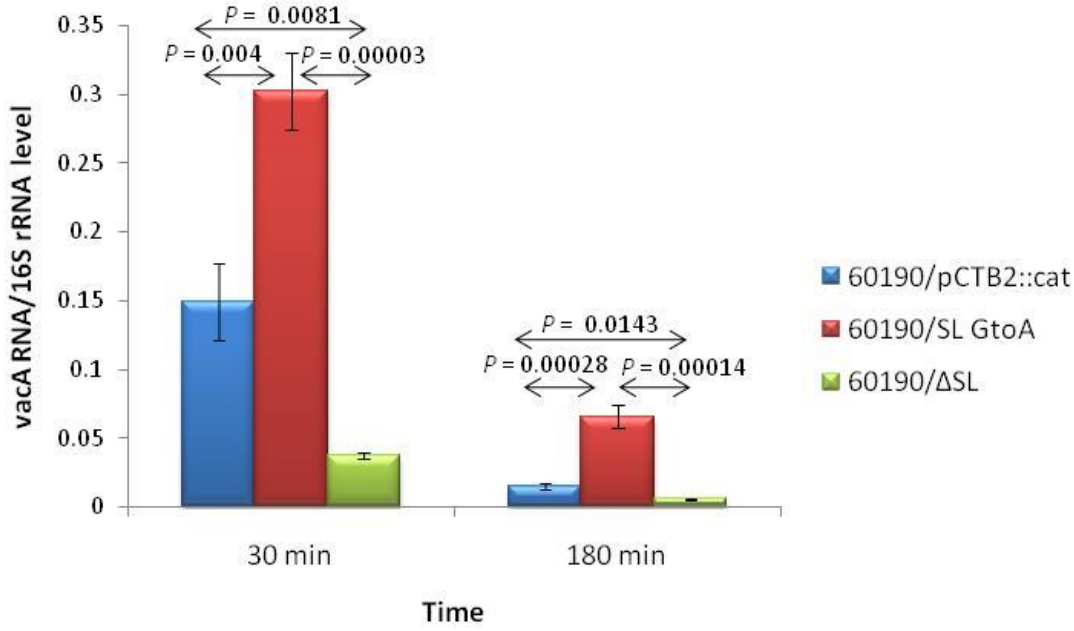


Figure 5.8 *vacA* mRNA levels of strains 60190/pCTB2::cat and 60190/ΔSL grown for 30 minutes and 180minutes in 10ml F12 Ham medium determined using quantitative real-time PCR. Mean *vacA* mRNA levels 60190/pCTB2::cat 30 minutes 0.149 (n=4), 180 minutes 0.015 (n=4); 60190/SL GtoA 30 minutes 0.3 (n=8), 180 minutes 0.066 (n=8). 60190/ΔSL 30 minutes 0.037 (n=4), 180 minutes 0.0059 (n=4). The difference between the means of two strains was determined by t-test: two sample assuming unequal variance (two-tail). Error bars show the standard error.

5.2.6 Disruption of the upstream inverted repeat has no effect on *vacA* mRNA levels.

To determine whether the presence of a perfect inverted repeat upstream of the -35 motif in the *cysS-vacA* intergenic region was important in determining differences in *vacA* levels between strains the first 6 nucleotides of the perfect inverted repeat were changed from AGCAAT to CATAGA as described previously in section 5.2.4 using the plasmid pCTB2::*cat* as a template. The same nucleotides were used but in a different order to maintain the similar Gibbs free energy. The mutant plasmids were introduced into *H. pylori* strain 60190 by natural transformation and double homologous recombination which was selected for by chloramphenicol resistance. The transformation of *vacA* into the correct location in the genome and the presence of the expected mutation and the absence of secondary mutations (Figure 5.9) was confirmed using the methodology described in section 5.2.4.

As the presence of a perfect inverted repeat was associated with high *vacA* mRNA levels *in vitro* strains two independent isolates of the inverted repeat mutant 60190/IRDIS (60190/IRDIS sc1 and sc4) and control strain 60190/pCTB2::*cat* were cultured for 18hrs in SFBB as detailed in section 2.2.5 and the level of *vacA* transcript determined using quantitative real-time PCR as described in section 4.3.3. Three separate cultures of each strain for each time point were used. Data from strains 60190/IRDIS sc1 and sc4 were combined to give a total number of six independent cultures of 60190/IRDIS. No significant difference in *vacA* mRNA levels was observed between the two strains with a

mean *vacA* transcript level of 0.144 and 0.125 for 60190/pCTB2::*cat* and 60190/IRDIS respectively (Figure 5.10).

```
                    10                  20                  30
G C T T T T C A A A T T T T A C C T T T T T A C A C A T T C  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc1
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc4
```

Inverted Repeat

```
                    40                  50                  60
T A G C C A A A A A T T C T A G C A A T A T T G C T T T T T  60190/pCTB2::cat
. . . . . . . . . . . . . . C A T . G A . . . . . . . . .  60190/IRDIS Sc1
. . . . . . . . . . . . . . C A T . G A . . . . . . . . .  60190/IRDIS Sc4

                    70                  80                  90
A A T C T T G T T G A G T T T T A T G T T T A T T T A C C T  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc1
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc4

                    100                 110                 120
T A A T T T G A T A A A A G T T T A A T A T T G G T T G T A  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc1
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc4

                    130                 140                 150
G A T A C T G C A T A T T T A T A G C C T T A A T C G T A A  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc1
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc4

                    160                 170                 180
A T G C A A C A G A A A T T T T C T A G T T T A A A G T C G  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc1
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc4

                    190                 200                 210
C A C C C T T T G T G C A A A A A A T T G T T T T A C A A G  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc1
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc4

                    220                 230                 240
A A A A G A A G A A A G G A A A G A A A T G G A A A T A C A  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc1
. . . . . . . . . . . . . . . . . . . . . . . . . . . . .  60190/IRDIS Sc4
```

Figure 5.9 The disrupted inverted repeat mutant 60190/IRDIS and the control strain 60190/pCTB2::cat were sequenced using primer A1043. Sequences were aligned using the MegAlign program (DNAStar). The inverted repeat is highlighted in blue. Mutated nucleotides are highlighted in pink.

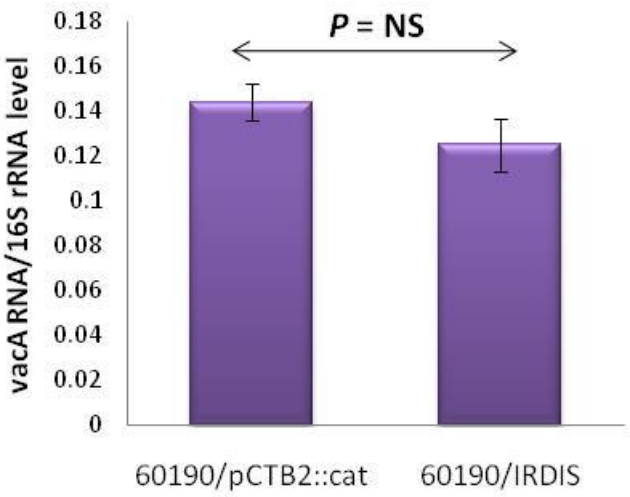


Figure 5.10 *vacA* mRNA levels of strains 60190/pCTB2::cat and 60190/IRDIS grown for 18 hours in broth culture determined using real-time PCR. Mean *vacA* mRNA levels of 0.144 and 0.125 for 60190/pCTB2::cat (n=4) and 60190/IRDIS (n=8) respectively. The difference between the means of two strains was determined by t-test: two sample assuming unequal variance (two-tail). Error bars show the standard error.

5.2.7 Search for transcription factor binding sites within the *cysS-vacA* intergenic region

A TRANSFAC Match™ search was carried out using the sequences of the intergenic regions of the 33 strains investigated in chapter 4 and on an extended sequence of 11 strains which consisted of a 771 bp region including the 3' end of *cysS* the *cysS-vacA* intergenic region and the region encoding the first 371bp of the translated 5' end of *vacA* (Figure 5.11). The TRANSFAC Match™ program contains data on known transcription factors and their experimentally proven binding sites, enabling a search for potential regulator binding sites in promoter sequences which have not yet been studied in detail. Only two strains (121 and 206) showed a match with a previously identified transcription factor binding site. The predicted binding site was 26 nucleotides in length and spanned the -35 region. However the sequence match was that of transcription factor CRP (cyclic AMP receptor protein also called CAP for catabolite activator protein) for which there is no known homologue in *H. pylori* suggesting that this was not a reliable match.

Inverted repeats have also been shown to act as sites for genetic rearrangements mediated by misalignment and can cause duplications or deletions which include the repeat itself and any intervening sequence. Perfect inverted repeats can arise when one copy of a small, imperfect, inverted repeat "corrects" another copy of the inverted repeat via strand misalignment (Lovett, 2004). To investigate whether the perfect repeats present in strains 192, 206 and 60190 had arisen by strain misalignment. The GeneQuest program (DNAStar) was used to search for other copies of imperfect versions of the

inverted repeat within the extended 771 bp sequence of 11 clinical strains. No other inverted repeats were observed within the sequence available. However a 63 bp deletion was observed in 6 out of 11 strains 18 bases upstream of the inverted repeatFigure 5.11). Direct repeats were observed directly upstream of the deleted region. The same direct repeat was observed 41 bases downstream of the first direct repeat in strains where the 63 bp region was still present indicating that this region had been lost through misalignment of the two repeats. Deletion of this 63 bp region did not correlate with *vacA* transcript levels *in vivo and in vitro*.

```
                                                  Direct repeat 1
A C G C C T C C A A T T T T A C C T T T T T A C A C A T T C   Majority
                    250                 260                 270
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   233
. . . . . . . . C . . . . . . . . . . . . . . . . . . . . .   256
G . T T T . . A . . . . . . . . . . A . . . . . . . . . . .   121
. . . . . . . . C . . . . . . . . . . . . . . . . . . . . .   249
G . T T T . . A . . . . . . . . . . . . . . . . . . . . . .   206
. . . . . . . . C . . . . . . . . . . . . . . . . . . . . .   126
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . T   194
. . . . . . . . C . . . . . . . . . . . . . . . . . . . . .   242
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   255
G . T T T . . A . . . . . . . . . . . . . . . . . . . . . .   60190
. . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Tx30a

T A G C C A A A A - - - - - - - - - - - - - - - - - - - - -   Majority
                    280                 290                 300
. . . . . . . . . T T T T C A G T A A G A G A G G C T T T T   233
. . . . . . . . . T T T T C A G T A A G A G A G G T T T T T   256
. . . . . . C . . A T T C C A A T G A G A G A A G T T T T T   121
. . A . . . . . . T T T T C A G T A A G A G A G G C T T T T   249
. . . . . . C . . A T T C C A A T G A G A G A A G T T T T T   206
. . . . . C . . . - - - - - - - - - - - - - - - - - - - - -   126
. . . . . . C . . - - - - - - - - - - - - - - - - - - - - -   194
. . . . . C . . . - - - - - - - - - - - - - - - - - - - - -   242
. . . . . . C . . - - - - - - - - - - - - - - - - - - - - -   255
. . . . . . . . . - - - - - - - - - - - - - - - - - - - - -   60190
. . . . . C . . . - - - - - - - - - - - - - - - - - - - - -   Tx30a

                                                  Direct Repeat 2
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Majority
                    310                 320                 330
T G A G C T T T T C A A A T T T T A C A T T T T T A C A C A   233
T A A G C T T T T C A A A T T T T A C A T T T T T A C A C A   256
T A A A C A C C T C C A A T T T T A C C T A T T T A C A C A   121
T A A G C T T T T C A A A T T T T A C A T T T T T A C A C A   249
T A A A C G C C T C C A A T T T T A C C T A T T T A C A C A   206
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   126
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   194
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   242
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   255
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   60190
- - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Tx30a

- - - - - - - - - - - - - A T T T C A G C A A T T T T A C T T   Majority
                    340                 350                 360
T T C T A G C C C A A A . . . . . . . . . . . . . . . . . .   233
T T C T A G C C C A A A . . . . . . . . . . . . . . . . . .   256
T T C T A A C C A C A A . . . C T . . . . . . A . . G . . .   121
T T C T A G C C C A A A . . . . . . . . . C . . . . . . . .   249
T T C T A A C C A T A A . . . C T . . . . . . A . . G . . .   206
- - - - - - - - - - - - . . . . . . . . . . . . . . . . . .   126
- - - - - - - - - - - - C . . . . . A T . T . . . . G . . .   194
- - - - - - - - - - - - . . . . . . . . . C . . G . . G . . .   242
- - - - - - - - - - - - . . . . . . . . . C . . G . . . . . .   255
- - - - - - - - - - - - . . . C T . . . . . . A . . G . . .   60190
- - - - - - - - - - - - . . . . . . . . . C . . . . . . . .   Tx30a
```

Figure 5.11 Direct repeats were observed directly upstream of the 63 bp deleted region. The same direct repeat was observed 41 bases downstream of the first direct repeat in strains where the 63 bp region had not been deleted. The extended *cysS-vacA* intergenic regions were sequenced using primer A1043. Sequences were aligned using the MegAlign program (DNAStar). The upstream direct repeat is highlighted in yellow and the downstream repeat is highlighted in pink. A dot represents a match with the consensus sequence and a dash represents the absence of a nucleotide.

5.3 Discussion

The control of mRNA stability is an important mechanism for controlling expression of many prokaryotic genes by controlling availability of transcripts for translation into protein (Emory *et al*., 1992, Hambraeus *et al*., 2000, Bechhofer and Dubnau, 1987, Carrier and Keasling, 1997b, Carrier and Keasling, 1997a). *H. pylori* has been shown to utilise mRNA decay to regulate the expression of the urease gene cluster in response to acidic pH (Akada *et al*., 2000). mRNA degradation in bacteria appears to be initiated by one or more endoribnucleolytic cleavages followed by digestion of the cleavage products by 3' to 5' exoribonucleases (Cannistraro *et al*., 1986, Emory and Belasco, 1990). Six ribonucleases are known to be involved in mRNA decay in *E. coli*, three of which are site specific endoribonucleases: RNase E, RNase G and RNase III. The other three are exoribonucleases: RNase II, polynucleotide phosphorylase and oligoribonuclease.

mRNA degradation in *E. coli* is primarily mediated by the single strand specific endoribonuclease RNase E (Ehretsmann *et al*., 1992a, Ehretsmann *et al*., 1992b, McDowall *et al*., 1994). RNase E requires a free single stranded 5' RNA region to bind to the target mRNA before scanning the transcript for cleavage sites which are substrates that lie 5' to AU dinucleotides in single-stranded segments (Mackie, 1991, Mackie and Genereaux, 1993, Ehretsmann *et al*., 1992a, Ehretsmann *et al*., 1992b, Emory and Belasco, 1990). RNase E cleavage is considered to be the rate limiting step in mRNA degradation (Kushner, 2002). Stem-loop structures located in the 5' UTR of many

prokaryotic transcripts have been found to protect mRNA from degradation by inhibiting RNase E binding to the 5' end of the transcript (Belasco *et al*., 1986, Bouvet and Belasco, 1992, Ehretsmann *et al*., 1992a, Ehretsmann *et al*., 1992b, Emory *et al*., 1992, Hansen *et al*., 1994) thus preventing the 3' to 5' digestion of cleavage products by exoribonucleases. The half life of the normally labile *blaA* transcript was increased by the addition of a stem-loop structure to the 5' terminal end of the transcript (Georgellis *et al*., 1992). Deletion of the 5' stem-loop of the *ompA* transcript reduces its half-life by a factor of 5 (Emory *et al*., 1992). In the absence of the *ompA* 5' stem-loop, degradation was found to start downstream of the 5' end (Emory *et al*., 1992). Several features of the 5' stem-loop structures appear to be important in determining transcript stability: the length of the stem, the size of the loop and the folding energy of the stem-loop (Bechhofer and Dubnau, 1987). Mutational analysis by Emory *et al.*, (1992) also revealed that the length of the single stranded region is crucial for mRNA stability, as the presence of a single stranded region of more than 4 nucleotides at the 5' terminus reduced mRNA stability (Emory *et al*., 1992). However it has been suggested that the optimum position of the stem-loop could be strain specific, as some bacteria have stem-loops at distances slightly further than 5 nucleotides from the extreme 5' end (Regnier and Arraiano, 2000).

Alternatively the increase in steady-state *vacA* mRNA levels could be due to a difference in RNase III. Endoribonuclease RNase III has been shown to remove the 5' stem-loops responsible for mRNA stability (Regnier and Arraiano, 2000). RNase III cleaves double stranded RNAs of low sequence complexity. Recognition of the substrate by RNase III is dependent on the

length of the double helical structure with a minimum length of one full turn or approximately 11bp (Court el al., 1993, Nicholson *et al*., 1999, Robertson *et al*., 1982). Watson-Crick base pair sequence elements also participate in controlling substrate reactivity. Two specific regions, a 3-bp proximal box (pb) and a 2-bp distal box (db) are involved in RNase III binding with the presence of certain Watson-Crick base pairs at specific positions within the proximal and distal boxes, strongly inhibiting cleavage by RNase III (Zhang and Nicholson, 1997, Li and Nicholson, 1996). Introduction of one or more of these excluded Watson-Crick base pairs into either the proximal or distal box in a model substrate inhibited cleavage by RNase III due to an interference in RNase III binding (Zhang and Nicholson, 1997). A possible explanation for the differences in *vacA* transcript levels seen between 60190/SL GtoA and the control strain 60190/pCTB2::*cat* could be that the presence of adenine rather than guanine at the +28 position within the 5' stem-loop of *vacA* creates an excluded Watson-Crick base pair which inhibits RNase III binding and cleavage. An RNase III sequence homologue has been identified in *H. pylori* (Tomb *et al*., 1997), although its role in determining *vacA* transcript stability remains to be determined.

vacA mRNA levels were significantly reduced in the stem-loop deletion mutant 60190/ΔSL by 55% (P = 0.008), 46% (P = 0.03) and 66% (P = 0.0024) at time points 20 minutes, 40 minutes and 180 minutes respectively when compared to strain 60190/pCTB2::*cat* (Figure 5.6). Strains 60190/ΔSL and 60190/pCTB2::*cat* were co-cultured with AGS cells. After 30 minutes and 22 hours 67% and 42% reductions in *vacA* transcript levels were observed in

strain 60190/ΔSL respectively when compared to that of the control strain 60190/pCTB2::*cat*. Oddly, no significant difference in *vacA* mRNA levels was seen at 3 hrs (Figure 5.5 (A)). *vacA* mRNA levels were reduced by 62% and 72% in strain 60190/ΔSL compared to strain 60190/pCTB2::*cat* at 30 minutes and 22 hours when *H. pylori* cells were cultured in F12 Ham medium without AGS cells (Figure 5.5 (B)). *vacA* mRNA levels were significantly reduced in the stem-loop deletion mutant 60190/ΔSL by 75% (P = 0.0081) and 61% (P = 0.0143) at 30 minutes and 180 minutes respectively when compared to strain 60190/pCTB2::*cat* (Figure 5.8). The increase and decrease in the *vacA* mRNA levels between the control strain 60190 pCTB2::*cat* and the mutant strains 60190 SL GtoA and 60190 SL respectively may represent a difference in mRNA stability between the strains. The *vacA* mRNA level in ΔSL may be due to an increase in stem-loop stability that protects from degradation by RNase E. Previous studies have found that the length of the stem structure, the size of the loop, the folding energy of the stem-loop and a single stranded region of no more than 4 nucleotides from the 5'terminus of the RNA segment were all factors of 5' stem-loops that affect mRNA stability (Emory and Belasco, 1990). The presence of the stem-loop is more important than its primary sequence to the activity of the *ompA* mRNA stabiliser. The location of this stem-loop is crucial to its stabilizing effect against RNase E.

Steady state levels of *vacA* mRNA were significantly higher in strain 60190/SL GtoA when grown in 10ml of F12 Ham medium for 30 minutes and 180 minutes, with a 2 fold (P = 0.004), and 4.4 fold (P = 0.00028) increase in *vacA* transcript levels respectively when compared to control strain

60190/pCTB2::*cat*. The increase in *vacA* mRNA level in strain 60190/SL GtoA may be due to an increase in stem-loop stability that protects the transcript from degradation by RNase E. Another possibility is that the presence of unfavourable Watson-Crick base pairing within the putative proximal box in strains with an A at the +28 position hinders binding of RNase III inhibiting cleavage of this stem-loop and thus increasing the stability of the transcript in these strains.

No RNase E homologues have been identified in *H. pylori*. However, there is no RNase E sequence homologue in the genome of *Bacillus subtilis* (Kunst *et al*., 1997) there is evidence for an RNase E like activity in this organism (Condon *et al*., 1997, Belasco *et al*., 1986). The C-terminal half of *E. coli* RNase E is not highly conserved evolutionarily (Kido *et al*., 1996). A significantly divergent *rnase E* gene in *H. pylori* may explain its lack of identification. Also, the possibility that the 5' UTR stem-loop is a binding site for a trans-acting factor cannot be excluded either. Results of a preliminary experiment suggest that the differences in *vacA* transcript levels seen between the wild-type and the mutant strains are due to mRNA stability. Liquid cultures of stem-loop mutant strains 60190/ΔSL and 60190/SL GtoA, and the control strain 60190/pCTB2::*cat* were split into two; rifampicin was added to one half of the culture to arrest transcription, and the other half was untreated. RNA was isolated from cultures at time intervals of 60 minutes and 120 minutes and the levels of *vacA* mRNA present per bacterial cell for each strain were assessed simultaneously using real-time PCR. The amount of *vacA* mRNA degradation in each of the strains was determined by calculating the percentage reduction in

the level of *vacA* mRNA in cultures with rifampicin added compared to the cultures without rifampicin. A 30% reduction in the level of *vacA* mRNA was observed in the wild-type control strain 60190/pCTB2::*cat*, whereas only a 6% reduction in *vacA* transcript level was observed in the strain 60190/SL GtoA indicating that the presence of an adenine rather than a guanine at position +28 within the stem-loop increased transcript stability. Deletion of the stem-loop reduced the stability of the *vacA* transcript with a 41% reduction in *vacA* transcript levels in the stem-loop deletion mutant 60190/ΔSL compared to the control. However, due to time constraints only one experiment was performed. Further replicate experiments would need to be carried out to confirm that differences in *vacA* transcript levels are truly due to differences in mRNA stability.

The effect of deletion of the 5' stem-loop was dependent on the growth conditions used. *vacA* mRNA levels were significantly reduced in 60190/ΔSL compared to the control when co-cultured with AGS cells or cultured in tissue culture medium. No reduction in *vacA* mRNA levels were seen when strain 60190/ΔSL was grown in SFBB supplemented with 5% FBS with shaking for 18 hours. When co-cultured with AGS cells or grown in 300µl of F12 Ham medium for 3 hours in a 24 well plate there was no reduction in *vacA* transcript levels in strain 60190/ΔSL compared to the control. Interestingly, when strains were cultured in 10ml of F12 Ham medium for 3 hours a 61% (P = 0.0143) reduction was observed. Differences in growth phase or nutrient availability may explain the differences in *vacA* transcript levels observed between the different culture conditions used in this study. Previous studies have shown that

vacA expression is regulated in response to growth phase (Forsyth and Cover, 2000, Boonjakuakul *et al*., 2004). Moreover, most known 5' mRNA stabilisers function only under specific environmental conditions. In rapidly growing *E. coli* cultures the 5' UTR can stabilise the *ompA* transcript resulting in a half life of 17 minutes. However the half life of *ompA* is growth rate regulated and falls to approximately 4 minutes in slow growing cells (Emory and Belasco, 1990). Stem-loop structures in the 5' UTR have been shown to function as growth rate regulated mRNA stabilisers in *Serratia marcescens* and *Enterobacter aerogenes* and appear to be highly conserved amongst enteric bacteria (Chen *et al*., 1991). The stability of the succinate dehydrogenase (SDH) and subtilisin transcripts of *Bacillus subtilis* are also growth rate dependent (Melin *et al*., 1989, Resnekov *et al*., 1990). Several groups have reported regulation of mRNA stability in response to nutrient deprivation. After 24 hours of total energy, carbon, nitrogen and phosphorus starvation the mRNA half life of *Vibrio angustum S14* increased 6 fold (Albertson *et al*., 1990). The *E. coli ompA* and *blaA* transcripts show increased stability when cultures are moved from aerobic to anaerobic conditions during slow growth (Georgellis *et al*., 1992, Georgellis *et al*., 1993). mRNA stability plays a prominent role in the control of gene expression in response to cold shock; the *cspA* transcript in *E. coli* is unstable at 37˚C however when temperatures are reduced to 15˚C its stability increases from 12 seconds to 20 minutes (Brandi *et al*., 1996, Goldenberg *et al*., 1996, Fang *et al*., 1997).

Interestingly, *vacA* mRNA levels in both strains 60190/pCTB2::*cat* and 60190/ΔSL were approximately 70% higher at 30 minutes when strains were

co-cultured with AGS cells than without. This is in agreement with previous studies that also showed that *vacA* expression is up regulated upon binding to epithelial cells (Gieseler *et al*., 2005, Szczebara *et al*., 1999, van Amsterdam *et al*., 2003). An increase in *vacA* transcript levels has also been observed during infection of PMNs (Gieseler *et al*., 2005). However the expression patterns were dependent on the infecting strain and were also dependent on time (Gieseler *et al*., 2005).

Narayanan, (2005) investigated *vacA* mRNA stability in the stem-loop deletion mutant 60190/ΔSL and the control strain 60190/pCTB2::*cat.* RNA was isolated from cultures at time intervals after transcription inhibition with rifampicin and *vacA* transcript levels were assessed by Northern blot. *vacA* transcripts in 60190/ΔSL were less stable than *vacA* transcripts in strain 60190/pCTB2::*cat.* However there were no detectable differences in VacA protein levels between the two strains. In this study when strains 60190/ΔSL and 60190/pCTB2::*cat* were cultured for 18 hours in SFBB no detectable difference in *vacA* transcript levels or protein levels were observed. The disparity between the two studies could possibly be explained by the culture conditions used in the two studies; Narayanan (2005) analysed cultures treated with rifampicin when investigating transcript levels between the two strains, separate 24 hour broth cultures with no rifampicin were used when protein levels were determined. It may be that when strains are rapidly growing (e.g. in 24 hour broth culture in complex media), the effect of differences in mRNA stability may not be apparent. Treatment with rifampicin blocks transcription and as a result growth will also be severely impaired. It may be that the effect of the differing mRNA stability

between the two strains would be detectable only in this state of metabolic stress.

Figure 4.6 in chapter 4 shows that strains with a fully palindromic inverted repeat express significantly more *vacA* than other strains (*P*= 0.01). No difference in *vacA* transcript levels were observed between the control strain 60190/pCTB2::*cat* and 60190/IRDIS indicating that this region was not important in determining *vacA* transcript levels *in vitro*. The significant association seen in chapter 4 may be due to changes outside the inverted repeat, furthermore, the sequence of the inverted repeat does not match any known regulator binding sites. A TRANSFAC Match™ search carried out on the *cysS-vacA* intergenic sequence revealed a match to a CRP binding site in strains 121 and 206 which was 26 nucleotides in length and spanned the -35 motif. However no CRP homologue has been identified in *H. pylori* and may represent a false positive result.

Inverted repeats have also been shown to act as sites for genetic rearrangements mediated by misalignment, and can cause duplications or deletions which include the repeat itself and any intervening sequence (Lovett *et al*., 2004). Perfect inverted repeats can arise when one copy of a small, imperfect, inverted repeat corrects another copy of the inverted repeat via strand misalignment (Lovett *et al*., 2004). No other inverted repeats were observed within the 771 bp sequence including the 3' end of *cysS*, the *cysS-vacA* intergenic region and the 5' end of the *vacA* coding sequence. This would suggest that the perfect inverted repeat in strains 60190, 206 and 121 had not

arisen through strand misalignment between two imperfect inverted repeats. A 63 bp deletion was observed in 6 out of 11 strains 18 bases upstream of the inverted repeat. Direct repeats were observed directly upstream of the deleted region: the same direct repeat was observed 41 bases downstream of the first direct repeat in strains where the 63 bp region was still present, indicating that this region had been lost through misalignment of the two repeats. Deletion of this 63 bp region did not correlate with *vacA* transcript levels *in vivo* or *in vitro* which would suggest that this region is not important in determining *vacA* transcript levels. Garner *et al*. (1995) also observed that a 63bp deletion occurred in 3 out of 6 strains analysed. They also found that the presence or absence of this 63bp region was not associated with the cytotoxin phenotype in these strains (Garner and Cover, 1995).

5.3.1 Conclusions

The presence of the stem-loop in the 5' UTR of *vacA* is important in determining steady-state levels of *vacA* transcript. Replacing a G with an A at position +28 a substitution present in wild-type clinical strains, resulted in an increase in the level of *vacA* mRNA. This finding is in agreement with the observation in chapter 4, which showed the presence of an A at the +28 position was significantly associated with higher levels of *vacA* transcript in clinical strains *in vivo*. In agreement with previous studies, *vacA* expression was up-regulated when bound to AGS cells. No difference in *vacA* mRNA levels were observed between the stem-loop deletion mutant and the control when strains were cultured in SFBB, but differences in *vacA* expression were

observed when cultured in tissue culture medium which may indicate that *vacA* expression is either dependent on growth phase or the differing environmental or nutritional factors between the two different types of culture media used. Preliminary results suggest that the differences in *vacA* transcript levels observed between the two stem-loop mutants and the control strain are due to differences in mRNA stability, however further studies would have to be carried out to confirm this finding and to determine the mechanism by which the 5' stem-loop regulates *vacA* transcript levels.

Figure 4.6 in chapter 4 showed that strains with a fully palindromic inverted repeat express significantly more *vacA* than other strains (P= 0.01). No difference in *vacA* transcript levels were observed between the control strain 60190/pCTB2::*cat* and 60190/IRDIS indicating that this region was not important in determining *vacA* transcript levels *in vitro*. The significant association seen in chapter 4 may be due to changes outside the inverted repeat. Furthermore, the sequence of the inverted repeat did not share significant sequence homology to known prokaryotic regulator binding sites. A 63 bp deletion, observed in 6 out of 11 strains 18 bases upstream of the inverted repeat appeared to be lost through misalignment of the two repeats. Deletion of this 63 bp region did not correlate with *vacA* transcript levels *in vivo* or *in vitro* which would suggest that this region is not involved in determining *vacA* transcript levels.

5.4 Future work

Preliminary results suggest that the differences in *vacA* transcript levels seen between the wild-type strain 60190/pCTB2::*cat* and the mutant strains 60190/ΔSL, 60190/SL GtoA are due to mRNA stability, However, further replicate experiments are required to determine whether this is reliable. Further studies are also needed to determine the mechanism of *vacA* mRNA degradation in *H. pylori*. One example might be to assess the involvement of RNase III in determining *vacA* transcript levels by knocking out *rnc* gene encoding RNase III in *H. pylori* and assessing its effect on *vacA* transcript levels.

Several studies have shown that acid exposure affects *vacA* transcription. These studies generally showed that *vacA* expression was induced under acidic conditions (Barnard *et al*., 2004, Merrell *et al*., 2003a, Choi *et al*., 2001). One study indicated that *vacA* expression was repressed after acid exposure at pH 5.0 (Gancz *et al*., 2006). Two other studies found no difference in *vacA* expression in response to acid exposure (Allan *et al*., 2001, Ang *et al*., 2001). *vacA* transcription has also been shown to be up regulated in response to iron depletion *in vitro* (Szczebara *et al*., 1999, Merrell *et al*., 2003). Other studies have shown that *vacA* transcript levels are increased by attachment to epithelial cells (van Amsterdam *et al*., 2003, Bach *et al*., 2002, Graham *et al*., 2002, Salama *et al*., 2000, Gieseler *et al*., 2005). Additionally, *vacA* expression has been found to be growth phase dependent (Thompson *et al*., 2003, Narayanan, 2005, Forsyth and Cover, 2000, Boonjakuakul *et al*., 2004). It would be

interesting to investigate whether the 5' stem-loop in *vacA* plays a role in regulating *vacA* production in response to these environmental conditions.

Chapter 6

6 Characterisation of the effect of polymorphic differences in the region encoding the VacA signal cleavage recognition site on VacA production.

6.1 Introduction

Many secreted bacterial proteins possess a signal sequence in their N-terminus, which directs secretion by the general secretion pathway. The signal peptidase then cleaves the signal sequence from the N-terminus of membrane and secreted proteins after they have been translocated across the cytoplasmic membrane. Bacterial signal sequences possess a positively charged N-terminus that orientates the signal in the inner membrane (N-region), a hydrophobic core that forms a membrane spanning helix (H-region), and a polar C-terminal region (C-region) that contains a cleavage recognition site that acts as a substrate for the leader peptidase. The C-region must have a β stranded formation to be recognised by the signal peptidase (Izard and Kendall, 1994). Analysis of a large number of signal sequences has shown there is a characteristic pattern of small apolar residues at positions -1 and -3 relative to the cleavage site. Alanine, glycine and serine are preferred at the -1 position. In addition to alanine, glycine and serine, larger residues such as valine, threonine, leucine and isoleucine can be accommodated at the -3 position. Such structural regularity in the -3 and -1 region is considered to be necessary for recognition of the signal peptide cleavage site by leader peptidase. A helix breaking residue is needed at position -6. It has been observed that these positions are the most important with respect to processing, with positions -2, -

4, -5 and +1 being of secondary importance (von Heijne, 1983, von Heijne, 1984, Perlman and Halvorson, 1983). However, the presence of proline at the +1 position has been shown to prevent cleavage of the signal peptide (Barkocy-Gallagher and Bassford, 1992, Nilsson and von Heijne, 1992).

The signal region of VacA is polymorphic. VacA type s1 and VacA type s2 not only differ in the position of their signal cleavage site, which determines their toxicity (with type s2 possessing a 12-amino acid hydrophilic N-terminal extension (Letley *et al*., 2003, Letley and Atherton, 2000), but also in the amino acids present at various positions within the signal sequence (Figure 6.1). Both VacA type s1 and s2 have alanine present at the -1 position. Studies on the processing of *E. coli* alkaline phosphatase, show that the presence of alanine, glycine and serine at the -1 position allows processing, with alanine providing the highest rate of processing (Karamyshev *et al*., 1998). Serine is present at position -3 in type s1 VacA, whereas type s2 VacA has leucine present at this position. Studies on the effect of sequence variation on the processing of the M13 procoat protein revealed that, only procoat proteins with small amino acids (serine and glycine) at the -3 position were efficiently cleaved *in vivo*, whereas presence of leucine at the -3 position retarded cleavage (Shen *et al*., 1991). However it has been suggested that the effect of some amino acid substitutions at position -3 on processing may vary from protein to protein, as mutant maltose-binding proteins with leucine, threonine or valine at the -3 position were processed efficiently (Fikes *et al*., 1990).

A characteristic feature of bacterial signal sequences is the presence of a helix-destabilizing residue such as a proline or glycine between the -4 and the -6 position of the peptide. In type s1 VacA, proline is present at -6, whereas in type s2 glycine is present at this position. Although proline and glycine are both effective helix breakers, procoat substituted with a glycine at the -6 position was not processed *in vivo*, and had reduced processing *in vitro*, compared to the wild type with proline at -6 (Shen *et al*., 1991). It is thought that proline helps alter the conformation of the polypeptide chain surrounding the cleavage site, allowing efficient cleavage of the signal peptide. In addition to the differences between VacA type s1 and s2 at the -1, -3, and -6 positions, it has also been observed that there are differences in the signal sequences between VacA type s1a strains that possess a glutamine at the -4 position and, type s1b VacA that has glutamate at -4 (Figure 6.1). However a study of the processing of mutant alkaline phosphatases showed that only lysine when present at the -4 position affects processing (Karamyshev *et al*., 1998).

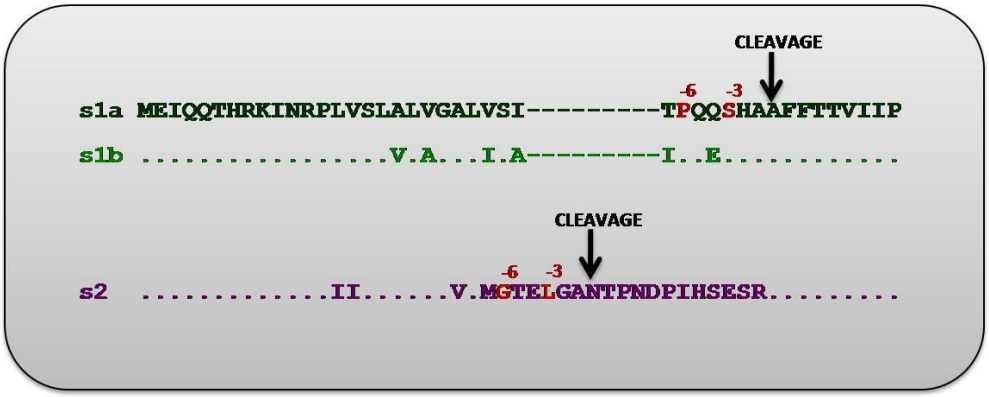


Figure 6.1 Signal sequences of VacA type s1a, s1b and s2. The cleavage site of each sequence is indicated by an arrow. Type s2 VacA possesses a 12-amino acid N-terminal extension and so differs in its cleavage site to s1 type *vacA*. All three signal sequences possess an alanine at the -1 position. Type s1a and s1b, VacA have a serine at position -3 and a proline at the -6 position, whereas type s2 VacA has leucine at position -3 and a glycine at position -6.

The presence of only type I signal peptidase is typical for many Gram-negative bacteria such as, *E. coli*, *Haemophilus influenzae*, *Pseudomonas fluorescens*, *Salmonella typhimurium* and *Yersinia pestis* (Dalbey and Wickner, 1985). The best characterised type I signal peptidase is that of *E. coli* which is also known as leader peptidase (Lep), and is encoded by the *lepB* gene (Wolfe *et al*., 1983, Zwizinski *et al*., 1981). The *E. coli* signal peptidase is a 35.9 kDa protein with 2 N-terminal transmembrane segments (residues 4-28 and 58-76) that enclose a small cytoplasmic domain (residues 29-57) which preceed the large C-terminal catalytic domain (residues 77-323). (Wolfe *et al*., 1983, Moore and Miura, 1987, San Millan *et al*., 1989). Bacterial signal peptidases are generally well conserved particularly in five regions known as boxes A, B, C, D, and E. Studies on the three dimensional structure of the *E. coli* signal peptidase Lep, revealed that it has predominantly β – sheet formation that consists of two anti-parallel β – sheet domains (Tschantz *et al*., 1995). Domain II contains box A which forms the N-terminal anchor. Domain I represents the catalytic domain which contains boxes B, C, D and E. All of these domains form part of or are close to the active site of the signal peptidase (See Figure 6.12.). The *E. coli* signal peptidase Lep has two small hydrophobic clefts that form the S1 and S3 binding pockets that accommodate the -1 and -3 residues of the signal peptide (Figure 6.11). The residues that form the S1 and S3 pockets are primarily hydrophobic. However, the S3 pocket is broader and not as deep as the S1 pocket which is consistent with the finding that larger residues can be accommodated at the -3 position of the signal peptide (Nielsen *et al*., 1997b, Nielsen *et al*., 1997a, Antelmann *et al*., 2001).

The amount of VacA produced by a strain has been shown to be important in *H. pylori* pathogenesis. In chapter 3 a significant positive association was found between the *vacA* mRNA level of an infecting strain and the severity of inflammation, activity, atrophy and infection density in the gastric mucosa. A small study carried out by Narayanan, (2005) showed that there was an association between high *vacA* transcript levels and the occurrence of ulcers or erosions in the patient. Additionally, it has been shown that large amounts of toxin produce greater damage in animal models (Ogura *et al*., 2000). Previous studies on bacterial signal sequences have shown that the primary structure affects processing efficiency and therefore the amount of protein secreted (von Heijne, 1983).

6.1.1 Aims

The aim of this chapter was to assess the importance of the natural polymorphisms in the VacA signal sequence on toxin production in the toxic strain 60190, and the non-toxic strain Tx30a. Sequence differences between the signal peptidase LepB of strains 60190 and Tx30a were also investigated.

6.2 Results

Isogenic *H. pylori vacA* mutants were constructed by D.P. Letley prior to this study, details of the mutations made and their construction is outlined below. Isogenic *H. pylori vacA* mutants were made in which serine had been replaced by leucine at position -3 (60190/S(-3)L) of the signal peptide, and proline had

been replaced by glycine at position -6 (60190/P(-6)G) in the VacA sequence of strain 60190. Double mutants in which both proline and serine at positions -6 and -3 were replaced by glycine and leucine respectively 60190/(P(-6)GS(-3)L) had also been constructed. Isogenic *vacA* mutants of *H. pylori* strain Tx30a in which leucine had been substituted for serine at position -3 (Tx30a/L(-3)S), and glycine had been replaced by proline at position -6 (Tx30a/G(-6)P) in the VacA sequence of strain Tx30a were also constructed. Again double mutants were also created in which both glycine and leucine at positions -6 and -3 were substituted for proline and serine respectively (Tx30a/G(-6)PL(-3)S).

The plasmid templates used for site-directed mutagenesis of *vacA* were pCTB2::*cat* for the 60190 mutants, and pA153::*cat* for the Tx30a mutants. Both pBluescript based vectors contained the 3' end of the upstream gene cysteinyl-tRNA synthetase (*cysS*) and the 5' 274 or 418 nucleotides of *vacA* respectively. In both cases a chloramphenicol acetyl transferase cassette (*cat*) derived from pBSC103 (Wang and Taylor, 1990) was inserted upstream of *vacA*, immediately 3' to *cysS* (Letley *et al.*, 2003). The codons that encode amino acids at positions -3 and -6 were mutagenised using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, UK). For each mutation, complementary primers between 33 and 36 nucleotides in length were designed to span the site that was to be mutated with the mutations included in the middle of the primer. To allow simple screening, mutations were designed to either introduce or remove a restriction endonuclease site. These primers were then used to replicate the wild-type plasmid templates, which incorporated the

mutation into the resulting plasmids. The mutagenesis reactions were treated with *Dpn*I restriction endonuclease which cleaves at the site $G^{me}ATC$ where the adenosine is methylated. Because the wild-type plasmid was purified from an *E. coli* strain that methylates the DNA it will be cleaved by the *Dpn*I enzyme, whereas the *in vitro* synthesised mutated plasmid is not methylated so therefore it is not cleaved by the enzyme. The *Dpn*I treated plasmids were then transformed into the *E. coli* strain DH5α, and chloramphenicol resistant transformants selected. This resulted in the enrichment of mutated plasmids due to the cleaved wild-type plasmid being unable to replicate. To identify mutated transformants, plasmid DNA was extracted and screened by restriction analysis using the restriction sites that were created or removed in the mutagenesis step. At this stage the mutations were confirmed by sequencing. The mutagenised plasmids were then introduced into their homologous *H. pylori* strains by natural transformation. Mutated *vacA* alleles were incorporated into the chromosomal *vacA* gene by double homologous recombination which was selected for by chloramphenicol resistance. The presence of the mutations was confirmed by restriction analysis. To control for the insertion of the chloramphenicol acetyl transferase cassette (*cat*) upstream of the *vacA* promoter, control strains 60190/pCTB2::*cat* and Tx30a/pA153::*cat* were also prepared by introducing the wild-type plasmids into their homologous strains, in the same manner. Previous VacA ELISA assays have revealed that introducing *cat* upstream of the *vacA* promoter region does not affect VacA levels for both strains used (Letley *et al.*, 2003), these strains were used as controls.

6.2.1 The mutations made by site directed mutagenesis in the signal region of *Helicobacter pylori* strains, 60190 and Tx30a were confirmed by sequencing.

To determine the presence of the appropriate mutations in the chromosomal *vacA* gene and the absence of any secondary mutations, a 651bp and a 578bp region including 220 bp upstream of the *vacA* start codon, the region encoding the VacA signal sequence and the first 371 and 358 base pairs of the region encoding the mature cytotoxin was PCR-amplified from genomic DNA of wild-type *H. pylori* strain 60190 and Tx30a, and each independent isolate of their isogenic *vacA* signal sequence mutant derivatives using the forward primer A1043 and the reverse primer VacR10. Two independent isolates of each mutant were sequenced. Both strands were sequenced using an Applied Biosystems 3100 Genetic analyser (Biopolymer Synthesis and Analysis unit, University of Nottingham, UK). Sequences retrieved were aligned using the MegAlign program (DNAstar). Sequencing confirmed the presence of the expected mutation (Figure 6.2 (60190) and Figure 6.3 (Tx30a)) and that no secondary mutations were present in the *vacA* sequence and the sequence upstream of *vacA*.

```
M E I Q Q T H R K I N R P L V S L A L V  Majority
                  10                  20
M E I Q Q T H R K I N R P L V S L A L V  60190
. . . . . . . . . . . . . . . . . . . .  60190/pCTB2::cat
. . . . . . . . . . . . . . . . . . . .  60190/S(-3)L (1)
. . . . . . . . . . . . . . . . . . . .  60190/S(-3)L (2)
. . . . . . . . . . . . . . . . . . . .  60190/P(-6)G (1)
. . . . . . . . . . . . . . . . . . . .  60190/P(-6)G (2)
. . . . . . . . . . . . . . . . . . . .  60190/P(-6)GS(-3)L (1)
. . . . . . . . . . . . . . . . . . . .  60190/P(-6)GS(-3)L (2)

G A L V S I T P Q Q L H A A F F T T V I  Majority
                  30                  40
G A L V S I T P Q Q S H A A F F T T V I  60190
. . . . . . . . . . . . . . . . . . . .  60190/pCTB2::cat
. . . . . . . . . . . L . . . . . . . .  60190/S(-3)L (1)
. . . . . . . . . . . L . . . . . . . .  60190/S(-3)L (2)
. . . . . . . G . . . . . . . . . . . .  60190/P(-6)G (1)
. . . . . . . G . . . . . . . . . . . .  60190/P(-6)G (2)
. . . . . . . G . . . L . . . . . . . .  60190/P(-6)GS(-3)L (1)
. . . . . . . G . . . L . . . . . . . .  60190/P(-6)GS(-3)L (2)

I P A I V G G I A T   Majority
                  50
I P A I V G G I A T   60190
. . . . . . . . . .   60190/pCTB2::cat
. . . . . . . . . .   60190/S(-3)L (1)
. . . . . . . . . .   60190/S(-3)L (2)
. . . . . . . . . .   60190/P(-6)G (1)
. . . . . . . . . .   60190/P(-6)G (2)
. . . . . . . . . .   60190/P(-6)GS(-3)L (1)
. . . . . . . . . .   60190/P(-6)GS(-3)L (2)
```

Figure 6.2 Sequence alignment of wild-type *H. pylori* strain 60190, and its isogenic VacA signal sequence mutant derivatives. Each strain was sequenced using the forward primer A1043 and the reverse primer VacR10. The appropriate mutation was confirmed by nucleotide sequencing on both strands using an Applied Biosystems 3100 Genetic analyser (Biopolymer Synthesis and Analysis unit, University of Nottingham, UK) Sequences retrieved were aligned using the MegAlign program (DNAstar). Mutants are named according to the wild type residue replaced, its position relative to the signal sequence cleavage site (shown in parentheses) and the residue substituted. The deduced amino acid sequence of the signal peptide and first 17 residues of mature VacA are shown.

```
M E I Q Q T H R K I N R P I I S L A L V  Majority
                  10                  20
. . . . . . . . . . . . . . . . . . . .  Tx30a
. . . . . . . . . . . . . . . . . . . .  Tx30a/pA153::cat
. . . . . . . . . . . . . . . . . . . .  Tx30a/L(-3)S (1)
. . . . . . . . . . . . . . . . . . . .  Tx30a/L(-3)S (2)
. . . . . . . . . . . . . . . . . . . .  Tx30a/G(-6)P (1)
. . . . . . . . . . . . . . . . . . . .  Tx30a/G(-6)P (2)
. . . . . . . . . . . . . . . . . . . .  Tx30a/G(-6)PL(-3)S (1)
. . . . . . . . . . . . . . . . . . . .  Tx30a/G(-6)PL(-3)S (2)

G V L M G T E L G A N T P N D P I H S E  Majority
                  30                  40
. . . . . . . . . . . . . . . . . . . .  Tx30a
. . . . . . . . . . . . . . . . . . . .  Tx30a/pA153::cat
. . . . . . . S . . . . . . . . . . . .  Tx30a/L(-3)S (1)
. . . . . . . S . . . . . . . . . . . .  Tx30a/L(-3)S (2)
. . . . P . . . . . . . . . . . . . . .  Tx30a/G(-6)P (1)
. . . . P . . . . . . . . . . . . . . .  Tx30a/G(-6)P (2)
. . . . P . . S . . . . . . . . . . . .  Tx30a/G(-6)PL(-3)S (1)
. . . . P . . S . . . . . . . . . . . .  Tx30a/G(-6)PL(-3)S (2)

S R A F F T T V I I   Majority
                  50
. . . . . . . . . . .   Tx30a
. . . . . . . . . . .   Tx30a/pA153::cat
. . . . . . . . . . .   Tx30a/L(-3)S (1)
. . . . . . . . . . .   Tx30a/L(-3)S (2)
. . . . . . . . . . .   Tx30a/G(-6)P (1)
. . . . . . . . . . .   Tx30a/G(-6)P (2)
. . . . . . . . . . .   Tx30a/G(-6)PL(-3)S (1)
. . . . . . . . . . .   Tx30a/G(-6)PL(-3)S (2)
```

Figure 6.3 Sequence alignment of wild-type *H. pylori* strain Tx30a, and its isogenic VacA signal sequence mutant derivatives. Each strain was sequenced using the forward primer A1043 and the reverse primer VacR10. The appropriate mutation was confirmed by nucleotide sequencing on both strands using an Applied Biosystems 3100 Genetic analyser (Biopolymer Synthesis and Analysis unit, University of Nottingham, UK) Sequences retrieved were aligned using the MegAlign program (DNAstar). Mutants are named according to the wild type residue replaced, its position relative to the signal sequence cleavage site (shown in parentheses) and the residue substituted. The deduced amino acid sequence of the signal peptide and first 17 residues of mature VacA are shown.

6.2.2 Mutagenesis of the signal peptide affects VacA production in *H. pylori* strain 60190.

The effect of replacing serine and proline found at positions -3 and -6 of the type s1 VacA signal sequence respectively with leucine and glycine that are present at these positions in the signal sequence of the type s2 strain was investigated. To do this, VacA levels of 24 hour broth culture supernatants of the control strain 60190 pCTB2::*cat* and the isogenic mutant strains 60190/S(-3)L, 60190/P(-6)G, and 60190/P(-6)GS(-3)L (between eighteen and twenty-two broths were analysed for each mutant) were analysed by ELISA using the primary anti-VacA p58 antibody, and the secondary antibody goat anti-rabbit IgG peroxidise conjugate (Sigma, UK). VacA amounts were expressed as ELISA A_{490} units, which were then corrected for the bacterial OD_{600} of the broth cultures (as described in section 2.7.8). Multiple broths were tested for each independent isolate for each mutant. Mean values for independent isolates were similar; hence the results for each independent isolate for each mutant were combined for subsequent analysis. Replacing serine with leucine at position -3 did not significantly reduce the amount of VacA present compared to the control strain 60190/pCTB2::*cat* (Figure 6.4). The amount of VacA in 24 hour broth culture supernatant was significantly reduced for the isogenic mutant strain 60190/P(-6)G compared with the control strain (reduction of 28% (P <0.05)). Substitutions at both the -6 and -3 positions in the isogenic mutant strain 60190/P(-6)GS(-3)L also resulted in a significant reduction of VacA present in the broth culture supernatant compared to the control (Reduction of 23% (P <0.05)), similar to that of the single 60190/P(-6)G mutation. The *vacA*

null mutant strain was also included to ensure that any antibody binding was specific.

To confirm the data obtained by ELISA, western blots were performed on the same broth culture supernatant samples used for ELISA. The samples were first corrected for bacterial broth culture OD_{600} and separated by SDS-PAGE. VacA was detected, again using the primary anti-VacA antibody anti-p58 and the secondary antibody, goat anti-rabbit IgG peroxidase conjugate (Sigma, UK) (details of the methodology are outlined in section 2.7.5). The western blots confirmed the results observed for the ELISA, with no reduction seen for the 60190 strains with a serine to leucine substitution at position -3 compared to the control. A reduction in the amount of VacA produced was seen for both isogenic mutant strains 60190/P(-6)G and 60190/P(-6)GS(-3)L. A representative western blot is shown in Figure 6.5.

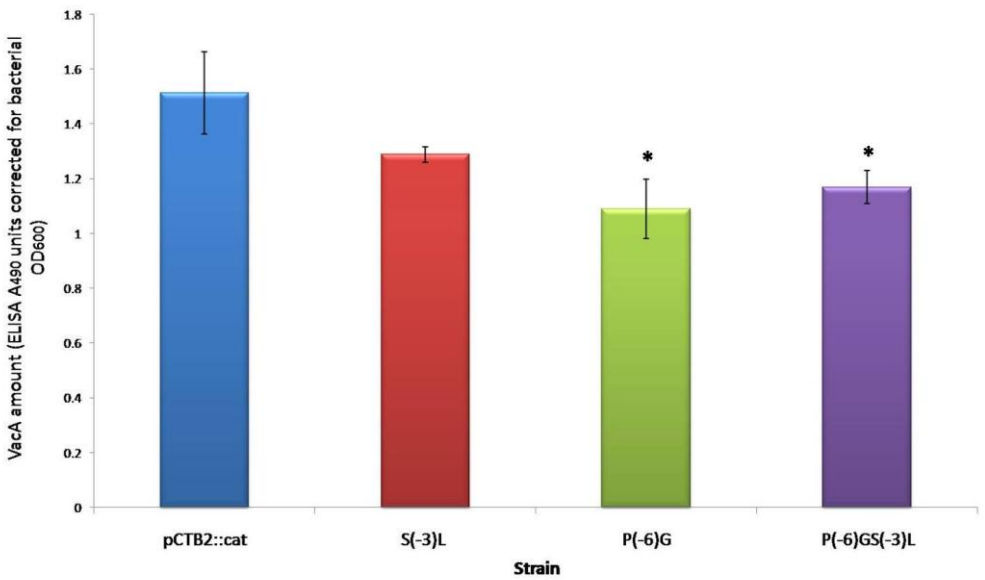


Figure 6.4 Mean VacA ELISA values for the control *H. pylori* strain 60190/pCTB2::cat and its isogenic VacA signal sequence mutant derivatives. Supernatants from 24hr broth cultures (a minimum of n=18) were analysed by ELISA and corrected for bacterial density OD600. Bars show mean amount of VacA produced by isogenic mutants of strain 60190. The error bars show the standard error. The significance of the difference in the mean of the control and mutant strains was determined using a T-test: two sample assuming unequal variance (two-tail) (* = P <0.05).

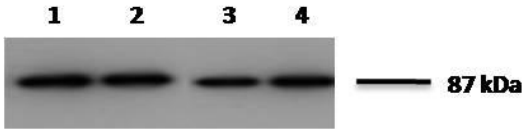


Figure 6.5 A representative VacA western blot of control strain 60190/pCTB2::cat and its isogenic VacA signal sequence mutant derivatives. 24 hour broth culture supernatants were separated by SDS-PAGE and immunoblotted with anti-VacA p58 antibody. Broth cultures were corrected to the same cell density and equal sample volumes were loaded as follows: Strains 60190/pCTB2::cat (lane 1), 60190/S(-3)L (lane 2), 60190/P(-6)G (lane 3), 60190/P(-6)GS(-3)L (lane 4). Samples are from non adjacent lanes of the same gel.

6.2.3 Mutagenesis of the signal peptide reduces VacA production in *H. pylori* strain Tx30a.

As the substitutions made in the 60190 isogenic mutants 60190/P(-6)G and 60190/P(-6)GS(-3)L resulted in a significant reduction in the amount of VacA present in the broth culture supernatants the reciprocal substitutions to the s2 signal sequence were also investigated. ELISAs to determine the amount of VacA produced by the Tx30a isogenic signal sequence mutants compared to the Tx30a/pA153::*cat* control were carried out in the same way as before, again using twenty-four-hour broth culture supernatants, (between thirteen and fourteen broths were analysed for each mutant) and using the primary anti-VacA antibody anti-p58 and the secondary antibody goat anti-rabbit IgG peroxidise conjugate (Sigma, UK). VacA amounts were expressed as ELISA A_{490} units, which were then corrected for the bacterial OD_{600} of the broth cultures. Replacing leucine with serine at position -3 significantly reduced the amount of VacA present in the cell culture supernatant compared to the Tx30a pA153::*cat* control by 54% (P <0.005) (Figure 6.6). The amount of VacA present in the 24 hour broth culture supernatant was also considerably reduced when glycine was replaced with proline at position -6, showing a reduction of 51% compared to the control (P <0.005). Substitutions at both the -6 and -3 positions further reduced VacA production, with a reduction of 77% compared to the control (P <0.005).

The results obtained using an ELISA were again confirmed by western blotting. The same broth culture supernatant samples used for the Tx30a ELISA were corrected for bacterial broth culture OD_{600} and separated by SDS-

PAGE. VacA was detected by western blotting, using the same primary and secondary antibodies as before. The results confirm the results observed for the ELISA, with reductions seen for both Tx30a strains with single mutations present at positions -3 and at -6. An even further reduction was seen for Tx30a strains with double mutations present at positions -3 and -6 of the VacA signal sequence (Figure 6.7).

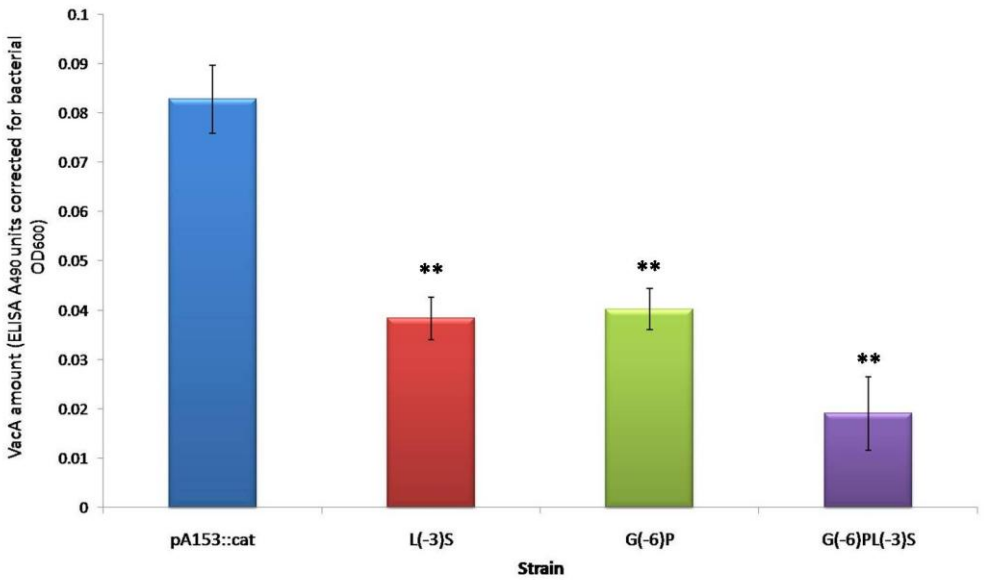


Figure 6.6 Mean VacA ELISA values for the control *H. pylori* strain Tx30a/pA153::cat and its isogenic VacA signal sequence mutant derivatives. Supernatants from 24hr broth cultures (a minimum of n=13) were analysed by ELISA and corrected for bacterial density OD600. Bars show mean amount of VacA produced by isogenic mutants of strain Tx30a. The error bars show the standard error. The significance of the difference in the mean of the control and mutant strains was determined using a T-test: two sample assuming unequal variance (two-tail) (= P <0.005).**

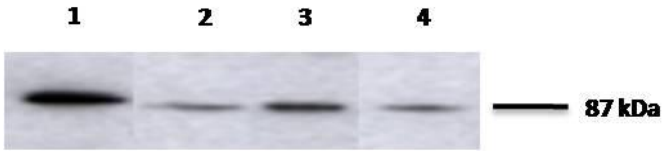


Figure 6.7 A representative VacA western blot of control strain Tx30a/pA153::cat and its isogenic VacA signal sequence mutant derivatives. 24 hour broth culture supernatants were separated by SDS-PAGE and immunoblotted with anti-VacA p58 antibody. Broth cultures were corrected to the same cell density and equal sample volumes were loaded as follows: Strains Tx30a/pA153::cat (lane 1), L(-3)S (lane 2), G(-6)P (lane 3), G(-6)PL(-3)S (lane 4). Samples are from non adjacent lanes from the same gel.

6.2.4 The mutant constructs pA153::*cat* and pA153::*cat* G(-6)PL(-3)S were transformed into *H. pylori* strain 60190 and the level of VacA secreted assessed.

To investigate whether it was the signal sequence or the strain background that was responsible for levels of VacA production, the mutant plasmid pCTB2::*cat* P(-6)GS(-3)L containing *vacA* from strain 60190 in which proline at the -6 position had been replaced with glycine and serine at the -3 position was replaced with leucine was transformed into *H. pylori* strain Tx30a, thus restoring the amino acids present at -3 and -6 to those normally present in Tx30a to assess whether VacA protein levels would be restored. The mutant plasmid pA153::*cat* G(-6)PL(-3)S containing *vacA* from strain Tx30a in which glycine at the -6 position had been replaced with proline, and leucine at the -3 position was replaced with serine was transformed into *H. pylori* strain 60190 by natural transformation to restore the amino acids present at -3 and -6 to those normally present in 60190. The mutant plasmids were introduced into their heterologous *H. pylori* strain backgrounds by natural transformation using the protocol described in section 2.5.2. The mutant *vacA* alleles incorporated into the chromosomal *vacA* gene by double homologous recombination. Strains that had incorporated the mutant *vacA* alleles were selected for by chloramphenicol resistance. Two independent isolates of strain 60190/pA153::*cat* G(-6)PL(-3)S were selected. To confirm that the insert had transformed into the correct location in the genome, DNA was isolated from both isolates and amplified using primers DL1 and C0374 that bind externally to the inserted region. PCR products amplified from strain 60190/pA153::*cat* G(-6)PL(-3)S had a product size of approximately 2000bp, whereas PCR

product amplified from the wild-type strain 60190 had a product size of approximately 900bp indicating that the insertion had transformed into the correct location. To confirm the presence of the correct mutation and the absence of secondary mutations a 578 bp region was PCR amplified from genomic DNA of the inverted repeat mutant 60190/pA153::*cat* G(-6)PL(-3)S using the forward primer A1043 and the reverse primer VacR10 then sequenced in both directions using primers A1043 and VacR10 using an Applied Biosystems 3100 Genetic analyser (Biopolymer Synthesis and Analysis unit, University of Nottingham, UK). Sequences retrieved were aligned using the MegAlign program (DNAstar). Sequencing confirmed the presence of the expected mutation and that no secondary mutations were present in the *vacA* sequence and the sequence upstream of *vacA.*

The effect of transforming the double mutant constructs into the heterologous strain background was investigated. To do this, VacA levels of 24 hour broth culture supernatants of the control strains 60190, 60190/pA153::*cat*, the *vacA* null mutant 60190/A130 and the isogenic mutant strain 60190/pA153::*cat* G(-6)PL(-3)S (three broths cultures of each strain were prepared for strain 60190/pA153::*cat* G(-6)PL(-3)S two broths of isolate sc1 and one broth of isolate sc2 were cultured.) were analysed by western blotting. The samples were first corrected for bacterial broth culture OD_{600} and separated by SDS-PAGE. VacA was detected, using the primary anti-VacA antibody anti-p58 and the secondary antibody, goat anti-rabbit IgG peroxidase conjugate (Sigma, UK) and the relative band density was determined by densitometry (Figure 6.9). A 27% reduction in VacA protein level was seen in strain 60190/pA153::*cat*

when compared to the control strain 60190. However this reduction was not significant (*P* = 0.25) (Mean band density 0.83 and 1.13 respectively). A significant reduction of 95% was seen for strain 60190/pA153::*cat* G(-6)PL(-3)S when compared to 60190 (*P* = 0.028) (Mean band density of 60190/pA153::*cat* G(-6)PL(-3)S 0.053). VacA levels were 94% lower for strain 60190/pA153::*cat* G(-6)PL(-3)S when compared to 60190/pA153::*cat* (*P* = 0.008).

```
M E I Q Q T H R K I N R P I I S L A L V  Majority

                  10                  20

M E I Q Q T H R K I N R P I I S L A L V  Tx30a
. . . . . . . . . . . . . . . . . . . .  60190/pA153::cat (1)
. . . . . . . . . . . . . . . . . . . .  60190/G(-6)PL(-3)S 1 (1)
. . . . . . . . . . . . . . . . . . . .  60190/G(-6)PL(-3)S 3 (2)

G V L M G T E L G A N T P N D P I H S E  Majority

                  30                  40

G V L M G T E L G A N T P N D P I H S E  Tx30a
. . . . . . . . . . . . . . . . . . . .  60190/pA153::cat (1)
. . . . P . . S . . . . . . . . . . . .  60190/G(-6)PL(-3)S 1 (1)
. . . . P . . S . . . . . . . . . . . .  60190/G(-6)PL(-3)S 3 (2)

S R A F F T T V I I P A I V G G I A T G  Majority

                  50                  60

S R A F F T T V I I P A I V G G I A T G  Tx30a
. . . . . . . . . . . . . . . . . . . .  60190/pA153::cat (1)
. . . . . . . . . . . . . . . . . . . .  60190/G(-6)PL(-3)S 1 (1)
. . . . . . . . . . . . . . . . . . . .  60190/G(-6)PL(-3)S 3 (2)

A A V G T V S G L L S W G L K Q A E Q A  Majority

                  70                  80

A A V G T V S G L L S W G L K Q A E Q A  Tx30a
. . . . . . . . . . . . . . . . . . . .  60190/pA153::cat (1)
. . . . . . . . . . . . . . . . . . . .  60190/G(-6)PL(-3)S 1 (1)
. . . . . . . . . . . . . . . . . . . .  60190/G(-6)PL(-3)S 3 (2)
```

Figure 6.8 Sequence alignment of wild-type *H. pylori* strain 60190, and its isogenic VacA signal sequence mutant derivatives. Each strain was sequenced using the forward primer A1043 and the reverse primer VacR10. The appropriate mutation was confirmed by nucleotide sequencing on both strands using an Applied Biosystems 3100 Genetic analyser (Biopolymer Synthesis and Analysis unit, University of Nottingham, UK) Sequences retrieved were aligned using the MegAlign program (DNAstar). Mutants are named according to the wild type residue replaced, its position relative to the signal sequence cleavage site (shown in parentheses) and the residue substituted. The deduced amino acid sequence of the signal peptide and first 17 residues of mature VacA are shown.

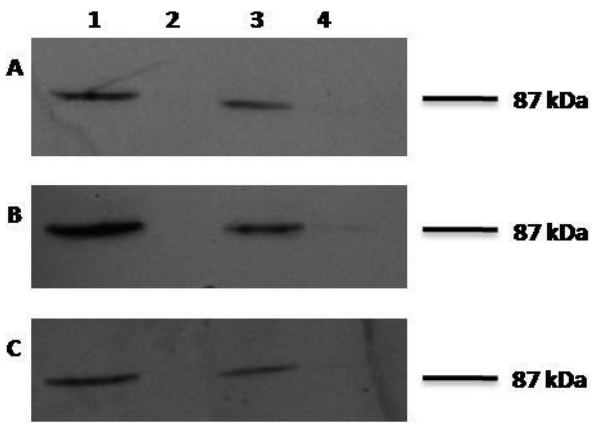


Figure 6.9 VacA western blots of strains 60190, 60190/pA153::cat and 60190/pA153::cat G(-6)PL(-3)S. 24 hour broth culture supernatants were separated by SDS-PAGE and immunoblotted with anti-VacA p58 antibody. Broth cultures were corrected to the same cell density and equal sample volumes were loaded as follows: 60190 (lane 1), A130 VacA null mutant (lane 2), 60190/pA153::cat (lane 3), and 60190/pA153::cat G(-6)PL(-3)S (lane 4).

6.2.5 Cell pellets of wild type and mutagenised *H. pylori* strains 60190 and Tx30a were analysed by western blotting.

Although the secretion pathway that VacA shares with autotransporter proteins predicts that mature VacA would be found in the culture supernatant, studies by (Fitchen *et al*., 2005), and (Ilver *et al*., 2004) have observed that up to 50 % of the secreted VacA associates with the bacterial cells that produce it. To ensure the difference in the amounts of VacA observed in the supernatant samples were actually due to a difference in production and not due to different amounts of VacA adsorbing to the *H. pylori* cell surface, the cell pellets of the broth cultures were analysed. The cell pellets of the broth cultures used to analyse the amount of VacA present in the cell culture supernatants were sonicated, corrected for bacterial OD_{600} and analysed by SDS-PAGE and western blotting. The western blots in Figure 6.10 show that there was no difference in the amount of VacA adsorbed to the surface of the *H. pylori* cells in the broth culture samples, and thus confirmed that the differences in the amount of VacA present in the cell culture supernatants seen was representative of the amount of VacA produced by the *H. pylori* strains.

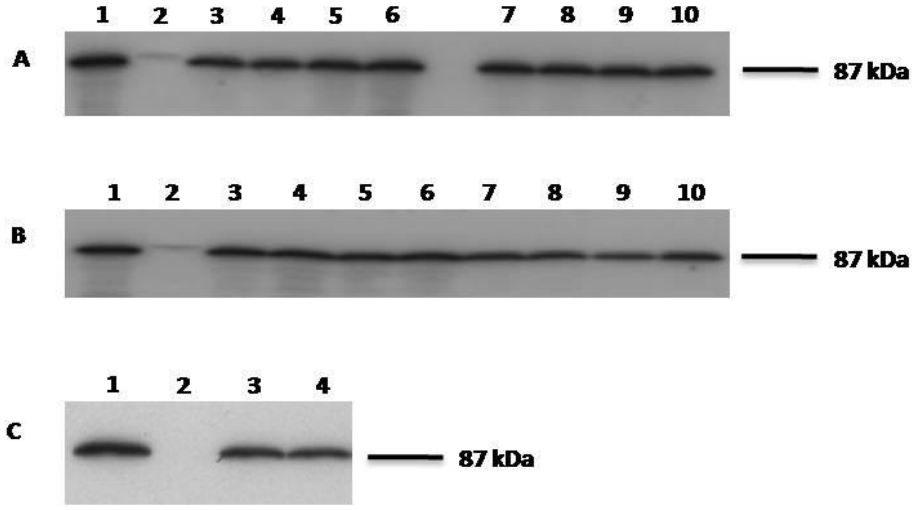


Figure 6.10 Bacterial cell adsorbed VacA levels of control strains and VacA signal sequence mutants. 24 hour broth culture whole cell extracts were separated by SDS-PAGE and immunoblotted with anti-VacA p58 antibody. Broth cultures were corrected to the same cell density and equal sample volumes were loaded as follows: Gel (A) 60190 wild-type (lane 1), A130 VacA null mutant (lane 2), 60190/pCTB2::cat (lanes 3 and 4), 60190/S(-3)L (lanes 5 and 6), 60190/P(-6)G (lanes 7 and 8), 60190/P(-6)GS(-3)L (lanes 9 and 10). Gel (B) Tx30a wild-type (lane 1), A130 VacA null mutant (lane 2), Tx30a/pA153::cat (lanes 3 and 4), Tx30a/L(-3)S (lanes 5 and 6), Tx30a/G(-6)P (lanes 7 and 8), Tx30a/G(-6)PL(-3)S (lanes 9 and 10). Gel (C) 60190 wild-type (Lane 1), A130 VacA null mutant (lane 2), 60190/pA153::cat (Lane 3), 60190/pA153::cat G(-6)PL(-3)S (Lane 4).

6.2.6 Could differences in the signal peptidase explain differences in signal peptide processing between strains 60190 and Tx30a

Although positions -6 and -3 appear to be important in determining secreted VacA levels, differences in processing between the 60190 and Tx30a do not appear to be solely due to the sequence of the signal peptide. Gram-negative bacteria commonly possess only one signal peptidase. The best characterised Gram-negative signal peptidase is that of *E. coli* (Lep encoded by the *lepB* gene). To investigate the importance of the signal peptidase determining signal sequence processing, the sequence of the signal peptidase was analysed in both 60190 and Tx30a.

6.2.7 BLAST search for *lepB* in 26695

The *lepB* sequence of *H. pylori* strain 26695 (*vacA* type s1/i1/m1) was used in a BLAST search against the *E. coli lepB* sequence. The BLAST results show that *H. pylori lepB* sequence from strain 26695 was 33% identical and 44% similar to that of *lepB* in *E. coli* (P =8 x 10^{-20}). The general topology is conserved although differences were seen in domains B-E and in the active site residues (Figure 6.12).

6.2.8 The LepB sequences of 60190 and Tx30a differ but not in the -1 and -3 cleavage recognition pockets.

The amino acid sequences of the LepB signal peptidase in *H. pylori* strains 60190 and Tx30a were analysed to determine whether they differed in the cleavage recognition pockets that accommodate the amino acids at positions -1,

-3 and in the cleavage recognition site. To do this primers were designed to amplify the a 300 base pair region of *lepB* containing sequence encoding the -1 and -3 cleavage recognition pockets using primers LepB forward and LepB reverse detailed in section 2.5.4.1 and amplified by PCR using cycling conditions detailed in sections 2.5.4. The PCR products sequenced using an Applied Biosystems 3100 Genetic analyser (Biopolymer Synthesis and Analysis unit, University of Nottingham, UK). Sequences retrieved were aligned using the MegAlign program (DNAstar). The sequence alignments show that the amino acid sequence of Lep differs between *H. pylori* strains 60190 and Tx30a at positions 189, 200 and 279. However, the regions that encode the S1 and S3 cleavage recognition pockets that accommodate the amino acids at positions -1, -3 in the signal peptide cleavage recognition site are conserved between the two strains (Figure 6.12).

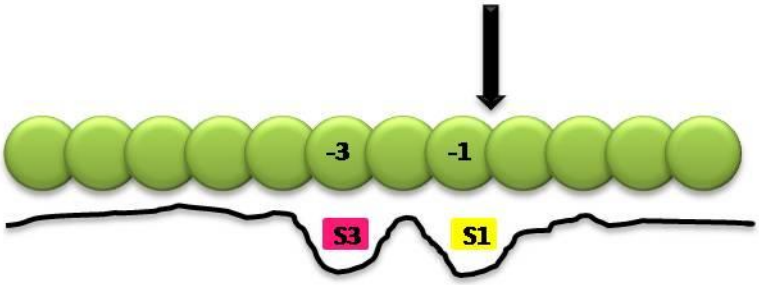


Figure 6.11 Diagram showing the location of the S3 and S1 binding pockets in relation to the -1 and -3 residues in the signal peptide cleavage recognition site. The black arrow indicates the site of cleavage. The S1 and S3 pockets are highlighted in yellow and pink respectively.

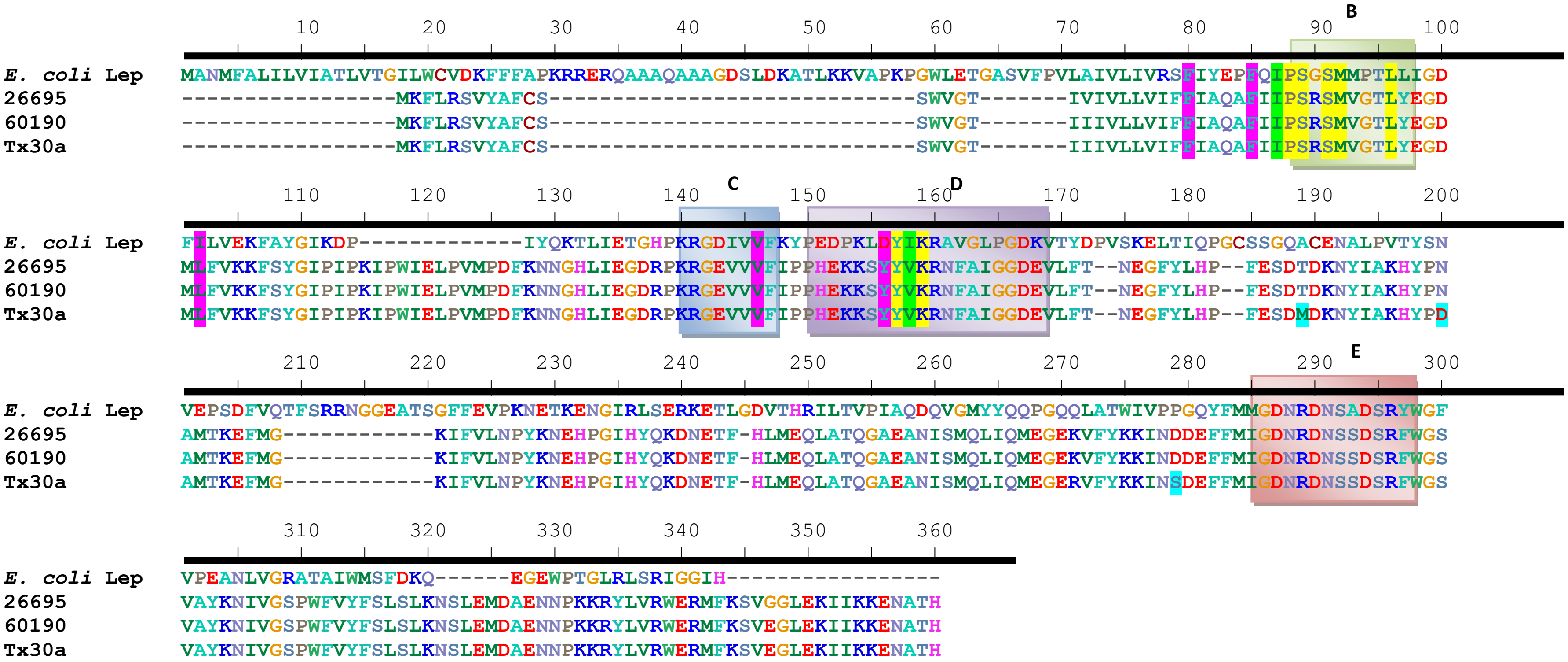


Figure 6.12 Sequence alignment of the deduced amino acid sequences of *E. coli* Lep and the *H. pylori* Lep homologue in strains 26695 (s1), 60190 (s1) and Tx30a (s2). Residues that form the S1 binding pocket are highlighted in yellow; residues of the S3 binding pocket are highlighted in pink and residues that form both the S1 and S3 binding pockets are highlighted in green. Amino acids that differ between strains 60190 and Tx30a are highlighted in blue. The sequence corresponding to the boxes B, C, D and E that form the catalytic domain are boxed. The Lep sequence of strains differ at positions 189, 200 and 279, however there are no differences within the S1 and S3 binding pockets.

6.2.9 The mutations made in the signal sequence of 60190 and Tx30a do not force cleavage to occur at another site

To investigate whether the mutations made in both the 60190 and Tx30a the signal sequence mutants create an alternative cleavage site SignalP analysis was carried out on the wild-type and mutant signal sequences. The SignalP software predicts the presence and location of signal peptide cleavage sites in amino acid sequences. To do this the amino acid sequences of the wild-type strains 60190 and Tx30a and their mutant derivatives were entered into the SignalP 3.0 Server http://www.cbs.dtu.dk/services/SignalP/. The Gram-negative bacteria organism group was selected and both the neural networks and hidden Markov models were used as the method of analysis. The analysis showed that replacing serine with leucine at the -3 position, and proline with glycine at the -6 position did not alter the signal peptide cleavage site when compared to that of the wild type signal sequence of 60190. There was also no change in the position of the signal peptide cleavage site when the double mutant 60190/P(-6)GS(-3)L was analysed. Similarly there was no change in the cleavage site compared to that of the wild-type Tx30a signal sequence when the Tx30a mutant strains Tx30a/G(-6)P, Tx30a/L(-3)S and Tx30a/G(-6)PL(-3)S were analysed. Thus, changing the amino acids present at positions -3 and -6 in the VacA signal recognition site of both strains 60190 and Tx30a appear to have no effect on the position of the signal peptide cleavage site. The location of the signal peptide cleavage sites of the VacA signal sequences of wild-type strains 60190 and Tx30a determined by SignalP analysis are in agreement with the positions of the cleavage sites of these strains determined

using N-terminal protein sequencing carried out by Atherton *et al.*, 1995 (see appendix for SignalP graphs showing the predicted location of the signal peptide cleavage sites for the 60190 and Tx30a signal sequence mutants).

6.3 Discussion

The aim of this chapter was to determine whether natural polymorphisms in the region encoding the VacA signal sequence affect toxin production, in the toxic strain 60190, and the non-toxic strain Tx30a. Firstly the effect of replacing the amino acids normally present in type s1 VacA with those amino acids present in type s2 VacA was characterised. The effect of the reciprocal changes in amino acids was also assessed. Secondly the effect of transforming the double mutant constructs into the heterologous strain background was investigated. Finally, the sequences of the LepB signal peptidase in *H. pylori* strains 60190 and Tx30a were analysed to determine whether they differed in the cleavage recognition pockets that accommodate the amino acids at positions -1 and -3 in the cleavage recognition site.

Investigation into the affect of changing serine and proline found at positions -3 and -6 of the type s1 VacA signal sequence with leucine and glycine respectively revealed that substituting serine for leucine at position -3 of the s1 VacA signal sequence did not significantly reduce the amount of VacA in the broth culture supernatant. This was surprising as previous studies (Shen *et al.*, 1991) have shown that the -1 and -3 positions are the most important with respect to processing, and investigations into the effect of sequence variation

on the processing of the M13 procoat protein revealed that only procoats with small amino acids (serine and glycine) at the -3 position were efficiently cleaved *in vivo*, whereas the presence of leucine at this position retarded cleavage (Shen *et al.*, 1991). However in support of the work in this report, it has been suggested that the effect of some amino acid substitutions at position -3 on processing may vary from protein to protein, as mutant maltose-binding proteins with leucine, threonine or valine at the -3 position were processed efficiently (Fikes *et al.*, 1990). Replacing proline with glycine at the -6 position showed a significant reduction in the amount of VacA secreted into broth culture (28% reduction). This result is in agreement with previous studies on signal peptides that show that the -6 position of the signal sequence is important for leader peptidase binding and catalysis. It has been proposed that signal peptides are efficiently processed when proline is preset at the -6 position as proline is able to break the α-helical hydrophobic segment of the leader peptide and bring the cleavage site of the signal sequence close to the active site of the leader peptidase. Substitutions at both the -3 and -6 positions in the double mutant 60190/P(-6)GS(-3)L resulted in a similar decrease in VacA secretion (Reduction of 23%) to that of the single proline to glycine mutant, this result is likely to reflect the effect of the proline to glycine substitution at the -6 position of the signal sequence. It could be speculated that these differences affect signal sequence processing efficiency. Although the results here do show that the primary sequence of the substrate is important for processing efficiency, it is still not sufficient to account for all the specificity of the signal peptidase. As the leader peptidase only cleaves precursor proteins some specific conformation of the substrate probably at the level of the

secondary and tertiary structure will be important for the substrate specificity of the leader peptidase (Shen *et al*., 1991).

The results obtained for the Tx30a mutants were surprising, as changing those amino acids normally present at the -3 and -6 positions in the VacA signal sequence of the non-toxic type s2 strain to those normally present at these positions in the signal sequence of the toxic s1 strain actually resulted in a reduction of secreted VacA. Previous studies on the effect of sequence variation on the processing of the M13 procoat protein revealed that only procoat proteins with small amino acids (serine and glycine) at the -3 position were efficiently cleaved *in vivo*, and the presence of leucine at the -3 position retarded cleavage (Shen *et al*., 1991). From this it was expected that replacing leucine with serine at the -3 position would result in an increase in VacA levels. However, replacing leucine with serine at position -3 resulted in VacA levels that were approximately half of that of the Tx30a/pA153::*cat* control strain (reduction of 54% (P <0.005)). This finding could be explained by the work of Fikes *et al.* 1990 who has suggested that the effect of some amino acid substitutions at position -3 on processing may vary from protein to protein, as mutant maltose-binding proteins with leucine, threonine or valine at the -3 position were processed efficiently (Fikes *et al*., 1990). The Tx30a/G(-6)P isogenic mutants also showed significantly reduced VacA production compared to the control showing a reduction of 51%. This was also a surprising result as although proline and glycine are both effective helix breakers, studies on the M13 procoat protein have shown that substitution of glycine at the -6 position prevented processing *in vivo*, and reduced processing

in vitro, compared to the wild type with proline at -6 (Shen *et al*., 1991). It is thought that proline helps alter the conformation of the polypeptide chain surrounding the cleavage site, allowing efficient cleavage of the signal peptide. The VacA levels seen for the double isogenic mutant Tx30a/G(-6)PL(-3)S reflected the sum of effects of the single mutations made at positions -3 and -6.

Transformation of the s2 signal sequence in which the amino acids at the -6 and -3 positions had been replaced with those normally found in s1 strains (pA153::*cat* G(-6)PL(-3)S) in to the s1 strain 60190 resulted in a 95% reduction in secreted VacA levels when compared to strain 60190. A 27% reduction was seen when the s2 signal sequence was transformed into the 60190 strain background. This suggests that the primary sequence including the amino acids adjacent to the -6 and -3 residues affects the efficiency of cleavage of the signal peptide. The order of the amino acids in the signal sequence are likely to affect the secondary and tertiary structure of the signal peptide which is likely to affect the specificity of the signal peptidase and therefore affect cleavage of the signal peptide.

Although the amino acid sequence of the putative *H. pylori* signal peptidase differed between the s1 and the s2 strains at 3 positions, the differences were not in the putative S1 and S3 binding pockets. Thus, the differences in VacA levels between strains 60190 and Tx30a are unlikely to be due to differences in the signal peptidase between strain 60190 and Tx30a.

The s1 type *H. pylori* strains have a signal peptide cleavage site that is more efficiently cleaved than s2 strains. It is possible that the amino acid differences in the s1 and s2 type signal sequences have arisen through mutation or recombination but have not been selected out because the s2 form of VacA is non-toxic.

The SignalP analysis suggests that the mutations made at the -6 and the -3 positions are unlikely to create an alternative signal peptide cleavage site. I tried to confirm this experimentally using N-terminal protein sequencing on the VacA isolated from broth cultures of the double mutant strains 60190 P(-6)GS(-3)L and Tx30a G(-6)PL(-3)S. Although VacA was detectable by Coomassie staining there was insufficient protein to get an accurate read.

6.4 Conclusions

In conclusion the work in this study has reinforced the findings of other studies that show the amino acids present at the -6 and -3 positions of signal peptide cleavage sites are important for the levels of protein secreted. The amino acids adjacent to the -6 and -3 positions also appear to be important for the levels of VacA produced. It is likely that changes to the primary structure of the signal sequence affects processing of the signal peptide, although the possibility that the mutations made may have an effect at other stages of protein production cannot be ruled out. No differences in the catalytic domains and S1 and S3 binding pockets of the putative *H. pylori* signal peptidase were observed between strain 60190 and Tx30a indicating that the differences in VacA levels

between strains 60190 and Tx30a are unlikely to be due to differences in the signal peptidase between strain 60190 and Tx30a.

6.4.1 Limitations

H. pylori has very specific growth requirements including an optimal temperature of 37°C, a carbon-dioxide rich 5% micro-aerophilic atmosphere, high humidity and a rich medium. To encourage similar rates of growth a starting OD_{600} of 0.1 at 600 nm was used for all broths as it has been reported that a higher inoculum reduces lag phase (Jiang and Doyle, 2000). Furthermore, all broths were corrected for optical density, to ensure that the amount of VacA detected was not due to differences in cell number. To further control for variation in growth, multiple broths were tested for each construct, and statistical analysis was used to take into account any variation. However even with these controls in place, the differences in growth for each broth cannot be ignored. This could be checked by growing further broths and taking a sample from each to determine the levels of *vacA* transcript by real time quantitative PCR to ensure that the result seen was a reflection of peptide processing efficiency and not differences in levels of *vacA* transcription.

Another source of error may have been in the construction of the signal sequence mutants, as *in vitro* synthesis of DNA could lead to secondary mutations due to lack of error-correction. To ensure this was not the case, the region mutagenised was sequenced. It is possible that other mutations may arise elsewhere in the genome during transformation and through passage of

the strains that could, possibly have an effect on VacA production. However, this is unlikely as duplicate isolates tested for each mutant showed similar levels of VacA. In addition the double mutants for both 60190 and Tx30a support the effect of the single mutants.

6.5 Future work

Transformation of pCTB2::*cat* and pCTB2::*cat* P(-6)GS(-3)L into the Tx30a strain background was unsuccessful. Given more time it would be interesting to see whether strain Tx30a/pCTB2::*cat* shows an increase in secreted VacA levels compared to Tx30a and whether strain Tx30a/pCTB2::*cat* P(-6)GS(-3)L shows reduced levels of secreted VacA compared to Tx30a/pCTB2::*cat*.

Chapter 7

7 Discussion

7.1 Summary of results and their significance

7.1.1 Genetic determinants of *vacA* production

Heterogeneity in *vacA* transcription between strains cultured *in vitro* was previously shown to exist and this difference in transcript level correlated with differences in VacA protein level (Forsyth *et al*., 1998; Forsyth *et al*., 1999; Ayala *et al*., 2004). *vacA* transcription has been shown to be initiated by a single promoter which is in the 285bp intergenic region located between the upstream housekeeping gene, cysteinyl-tRNA synthase (*cysS*) and the start of *vacA* (Forsyth *et al.*, 1998). The *vacA* promoter has a conserved -35 region (TTTATG), a conserved -10 region (TAAAAA) and a TGN motif adjacent to the -10 motif (Forsyth *et al.*, 1998). Using site directed mutagenesis and a *xylE* reporter system, Forsyth *et al.*, (1999) concluded that -35 and -10 sequences along with promoter element spacing are important for binding. However, two small studies carried out on small numbers of clinical strains *in vitro* found that the differences in *vacA* expression between strains were unrelated to naturally occurring polymorphic differences in the -10 and -35 sequences (Ayala *et al*., 2004; Narayanan, 2005).

The previous studies on the *vacA* promoter were carried out on a small number of strains cultured *in vitro* (Ayala *et al*., 2004; Narayanan, 2005) and the mutagenesis carried out by Forsyth *et al*., (1999) was random. Furthermore, Forsyth *et al.* did not investigate the polymorphic differences that occur naturally between clinical strains. This study investigated the effect of naturally

occurring differences in the *vacA* promoter on *vacA* production *in vivo* and *in vitro* in a large number of strains. Polymorphisms that were associated with significantly higher levels of *vacA* transcript *in vivo* and *in vitro* were further investigated using site directed mutagenesis.

In this study large variations in *vacA* mRNA levels between strains were observed *in vitro* and *in vivo*. In agreement with other studies differences in the *vacA* transcript level between the different strains *in vitro* and *in vivo* were not significantly associated with the polymorphic differences present within the -35 and extended -10 motifs. Polymorphic differences were observed in the spacer region between the -10 and the -35 motifs, however polymorphic differences in this region were not associated with differences in *vacA* mRNA levels between strains. Spacing of the -10 and -35 motifs in the clinical strains investigated in this study was well conserved.

In a previous study by Narayanan, (2005) using a sequence alignment of the published promoter regions of 19 *H. pylori* strains, Narayanan (2005) identified a conserved stem loop structure at the +4 position of the mRNA transcript. Deletion of this stem loop resulted in a reduction in *vacA* mRNA levels *in vitro*. The alignment also revealed that this stem loop was well conserved except for differences at the +11 and +28 positions within the stem loop. The importance of these polymorphisms in determining *vacA* transcript level was not determined at that time.

In this study it was found that strains naturally possessing an A at position +28 within the second stem of the stem loop were found to express more *vacA* than those that have a G at this position *in vivo* (P = 0.0089). Site directed mutagenesis where the G present at position +28 in the wild-type strain was replaced with an A resulted in an increase in the level of *vacA* mRNA.

The presence of varying numbers of binding sites for transcription factors could potentially explain the differences in *vacA* mRNA level between strains. Inverted repeats often represent binding sites for transcriptional regulators. In a small study by Ayala *et al*. (2004) an inverted repeat upstream of the -35 motif was found to be present in 4 out of 8 clinical strains (Ayala *et al.*, 2004). Inverted repeats are a common feature of regulator binding sites, however the role of the inverted repeat in the *cysS* – *vacA* intergenic region in transcriptional regulation of *vacA* was not determined.

In this study it was observed that strains possessing a perfect inverted repeat expressed significantly higher levels of *vacA in vitro*. However, when the perfect inverted repeat present in strain 60190 was disrupted using site directed mutagenesis no difference in *vacA* transcript levels were observed between the control strain and the mutant indicating that this region was not important in determining *vacA* transcript levels *in vitro*. Furthermore, the sequence of the inverted repeat did not share significant sequence homology to known prokaryotic regulator binding sites.

7.1.2 Environmental determinants of *vacA* expression

Several studies have shown that *vacA* is regulated in response to environmental stimuli. Microarray analysis demonstrated that *vacA* expression is repressed after acid exposure at pH 5.0 (Merrell *et al*., 2003a). This was in contrast to non-array based work which demonstrated that *vacA* was induced under acidic conditions (Barnard *et al*., 2004). Furthermore, RNA dot blot analysis revealed a significant increase in the accumulation of *vacA* transcripts under iron-restricted conditions (Szczebara *et al*., 1999). Other studies have shown that *vacA* expression is up regulated upon binding to epithelial cells (Gieseler *et al*., 2005; Merrell *et al*., 2003; Szczebara *et al*., 1999; van Amsterdam *et al*., 2003). An increase in *vacA* transcript levels has also been observed during infection of PMNs (Gieseler *et al*., 2005). However the expression patterns were dependent on the infecting strain and were also dependent on time (Gieseler *et al*., 2005). Previous studies have also shown that *vacA* expression is regulated in response to growth phase (Forsyth and Cover, 2000; Boonjakuakul *et al*., 2004).

Results obtained in this study showed that there was no correlation between *vacA* transcript levels of *H. pylori* strains *in vivo* when compared to the same strains cultured *in vitro* using matched strains and biopsies. Regions within the promoter associated with differences in *vacA* production between strains also differed *in vitro* and *in vivo*, which would suggest that *vacA* transcription is regulated *in vivo*, and could be regulated in response to growth phase of the bacterium or by environmental factors.

In agreement with previous studies, *vacA* expression was up-regulated when the wild-type and mutant *H. pylori* strains were co-cultured with AGS cells. No difference in *vacA* mRNA levels were observed between the stem-loop deletion mutant and the control when strains were cultured in SFBB, but differences in *vacA* expression were observed when cultured in tissue culture medium. This may indicate that *vacA* expression is either dependent on growth phase or the differing environmental or nutritional factors between the two different types of culture media used.

7.1.3 Regulation of production at the level of protein secretion

Analysis of a large number of signal sequences has shown there is a characteristic pattern of small apolar residues at positions -1 and -3 relative to the cleavage site. Alanine, glycine and serine are preferred at the -1 position. Larger residues such as valine, threonine, leucine and isoleucine can also be accommodated at the -3 position, although some studies have shown that presence of leucine at the -3 position retarded cleavage (Shen *et al*., 1991) (Fikes *et al*., 1990). Such structural regularity in the -3 and -1 region is considered to be necessary for recognition of the signal peptide cleavage site by a leader peptidase.

The presence of a helix breaking residue such as proline or glycine at the -6 position has also been shown to be important for processing (von Heijne, 1983, Perlman and Halvorson, 1983). Although proline and glycine are both effective helix breakers, some studies have shown that some signal peptides with glycine

at the -6 position are not processed *in vivo*, and had reduced processing *in vitro*, compared to the wild type with proline at -6 (Shen *et al*., 1991). It is thought that proline helps alter the conformation of the polypeptide chain surrounding the cleavage site, allowing efficient cleavage of the signal peptide.

The signal region of VacA is polymorphic. VacA type s1 and VacA type s2 not only differ in the position of their signal cleavage site, which determines their toxicity (with type s2 possessing a 12-amino acid hydrophilic N-terminal extension (Letley *et al*., 2003, Letley and Atherton, 2000), but also in the amino acids present at various positions within the signal sequence. Both VacA type s1 and s2 have alanine present at the -1 position.

Serine is present at position -3 in type s1 VacA, whereas type s2 VacA has leucine present at this position. In type s1 VacA, proline is present at -6, whereas in type s2 glycine is present at this position. In this study mutant strains in which the more favourably cleaved amino acids present at the -3 and -6 positions of the s1 strain 60190 were replaced with the less favourably cleaved amino acids present in the s2 strain Tx30a were characterised. The reciprocal changes were also made. Secondly the effect of transforming the double mutant constructs into the heterologous strain background was investigated. Finally, the sequences of the LepB signal peptidase in *H. pylori* strains 60190 and Tx30a were analysed to determine whether they differed in the cleavage recognition pockets that accommodate the amino acids at positions -1 and -3 in the cleavage recognition site.

Characterisation of these mutants showed that the amino acids present at the -6 and -3 positions of signal peptide cleavage sites is important for the levels of protein secreted. However, the amino acids adjacent to the -6 and -3 positions also appear to be important for the levels of VacA produced. It is likely that changes to the primary structure of the signal sequence affects processing of the signal peptide. No differences in the catalytic domains and S1 and S3 binding pockets of the putative *H. pylori* signal peptidase were observed between strain 60190 and Tx30a indicating that the differences in VacA levels between strains 60190 and Tx30a are unlikely to be due to differences in the signal peptidase between strain 60190 and Tx30a. The s1 type *H. pylori* strains have a signal peptide cleavage site that is more efficiently cleaved than s2 strains. It is possible that the amino acid differences in the s2 type signal sequences have arisen through mutation or recombination but have not been selected out because the s2 form of VacA is non-toxic.

7.1.4 VacA and disease

The particular *vacA* genotype of an infecting strain determines toxin production (Israel *et al*., 2001), and is a marker for the pathogenicity of an individual strain (Atherton *et al*., 1995; Rhead *et al*., 2007). Numerous studies have investigated the association between VacA type and disease outcome in different populations. *vacA* s1 genotypes are more frequently associated with higher levels of inflammation in the gastric mucosa, peptic ulceration and gastric adenocarcinoma than s2 types (Atherton *et al*., 1995; Atherton *et al*., 1997; Evans *et al*., 1998; Kidd *et al*., 1999; van Doorn *et al*., 1998; Rhead *et*

al., 2007; Basso *et al*., 2008). Corpus neutrophil infiltration was found to be more severe in patients infected with an m1 type strain than those infected with an m2 type strain (Umit *et al*., 2009). The *vacA* i1 type is a risk factor for peptic ulcer disease and has been shown to be associated with duodenal ulcer disease as well as gastric cancer (Basso *et al*., 2008; Rhead *et al*., 2007).

Several lines of evidence indicate that VacA may enable *H. pylori* colonization of the gastric mucosa. Competition experiments with an isogenic *vacA* null mutant and a wild type strain in a mouse model indicated that VacA may play a role in colonisation of the gastric mucosa (Salama *et al*., 2001). Salama *et al*. (2001) suggested that VacA causes tissue damage leading to alteration of the local pH and nutrient release allowing *H. pylori* to survive in the mucosa. Another suggestion was that cell damage caused by VacA plays a role in the local inhibition of acid secretion, which facilitates *H. pylori* colonisation (Salama *et al*., 2001). VacA has been shown directly damage the gastric epithelium, and it has been proposed that a gastric mucosal inflammatory response may occur as a consequence of the VacA mediated damage. Many previous studies provide evidence that VacA may be directly involved in proinflammatory signalling. (Boncristiano *et al*., 2003; Hisatsune *et al*., 2007; Supajatura *et al*., 2002; Kim *et al*., 2007).

In East Asian and Southeast Asian countries, the association between *vacA* allelic diversity and clinical outcome is not well established as the *vacA* allelic type is quite homogeneous, with the majority of the population being infected with type s1/i1 strains (Maeda *et al*., 1998; Yamaoaka *et al*., 1998). However,

it is possible that a high prevalence of the more virulent *vacA* s1/i1 type strains may contribute to the high incidence of gastric cancer in this region of the world (Rhead *et al*., 2007).

In a mouse model of infection oral administration of *H. pylori* sonicate from strains of the *vacA* s1 type reproduced several aspects of the histological lesions observed in biopsies from patients with gastric ulcer disease. These included epithelial vacuolation, infiltration of mononuclear cells into the lamina propria, loss of gastric gland structure and ulceration (Telford *et al*., 1994a, Ghiara *et al*., 1995). Sonicate of a non-toxigenic s2 strain did not induce histological lesions, indicating that the active s1 cytotoxin is necessary to induce gastric damage (Telford *et al*., 1994). Oral administration of purified VacA into the same mouse model induced the same histological lesions as those obtained with the crude extract, although inflammatory cell infiltration (Telford *et al*., 1994) and neutrophil infiltration was not observed (Supajatura *et al*., 2002). Interestingly gerbils infected with an isogenic *vacA* null mutant had significantly fewer ulcers when compared to those infected with an isogenic wild type toxigenic strain (Ogura *et al*., 2000). However the severity of gastritis in the gerbils infected with a null mutant was not significantly different from that seen in gerbils infected with the wild type strain (Ogura *et al*., 2000).

VacA amount as well as the type and activity is thought to have important implications for disease; a small study carried out by Narayanan, (2005) showed that there was an association between the occurrence of ulcers or

erosions in the patient and a high level of *vacA* transcript produced by an infecting strain *in vivo*.

In this study the association between the amount of *vacA* mRNA produced by a strain and the severity of pre-cancerous changes in the corpus of the patient were assessed using real-time PCR and histopathology. A significant positive association was seen between the level of *vacA* transcript produced per *H. pylori* cell and the severity of inflammation, activity and the presence of atrophy in the corpus of infected patients. A positive correlation between the *vacA* level of an infecting strain and the density of infection in the corpus was observed. However, no association was found between *vacA* transcript level and disease outcome as determined during the endoscopy.

7.2 Conclusions

In conclusion natural polymorphic differences within a stem loop at the start of the *vacA* transcript determines differences in *vacA* mRNA levels between clinical *H. pylori* strains *in vivo*. There is no correlation between the level of *vacA* mRNA produced by the same strain *in vivo* and cultured *in vitro*, implying that, in agreement with other studies, *vacA* transcription is regulated *in vivo*. Amino acid differences at the -6 and -3 positions of signal peptide cleavages sites between s1 and s2 strains are important for the levels of VacA secreted. However, the amino acids adjacent to the -6 and -3 positions also appear to be important for the levels of VacA produced. No differences in the catalytic domains and S1 and S3 binding pockets of the putative *H. pylori*

signal peptidase were observed between these strains indicating that the differences in VacA levels between strains 60190 and Tx30a are unlikely to be due to differences in the signal peptidase between strain 60190 and Tx30a. The s1 type *H. pylori* strains have a signal peptide cleavage site that is more efficiently cleaved than s2 strains. A significant positive association was seen between the level of *vacA* transcript produced per *H. pylori* cell and the severity of inflammation, activity and the presence of atrophy in the corpus of infected patients. A positive correlation between the *vacA* level of an infecting strain and the density of infection in the corpus was observed. However, no association was found between *vacA* transcript level and disease outcome as determined during the endoscopy.

7.3 Future work

7.3.1 Genetic determinants of *vacA* production

Preliminary results suggest that the differences in *vacA* transcript levels observed between the two stem-loop mutants and the control strain are due to differences in mRNA stability, however further studies would have to be carried out to confirm this finding and to determine the mechanism by which the 5' stem-loop regulates *vacA* transcript levels. One example might be to assess the involvement of RNase III in determining *vacA* transcript levels by knocking out the *rnc* gene encoding RNase III in *H. pylori* and assessing its effect on *vacA* transcript levels.

Several studies have shown that *vacA* is regulated in response to environmental stimuli such as pH (Barnard *et al*., 2004; Merrell *et al*., 2003; Choi *et al*., 2001; Gancz *et al*., 2006), depleted levels of iron (Szczebara *et al*., 1999, Merrell *et al*., 2003), the presence of epithelial cells (van Amsterdam *et al*., 2003; Kim *et al*., 2004; Bach *et al*., 2002; Graham *et al*., 2002; Salama *et al*., 2000; Gieseler *et al*., 2005) and the growth phase of the bacterium (Thompson *et al* 2003; Narayanan, 2005; Forsyth and Cover, 2000; Boonjakuakul *et al*., 2004). It would be interesting to investigate whether the 5' stem-loop in *vacA* plays a role in regulating *vacA* production in response to these environmental conditions.

The urease gene cluster has been shown to be regulated post-transcriptionally by mRNA decay in response to environmental pH (Akada *et al*., 2000). Together with the finding that *H. pylori* has relatively few transcriptional regulators (Scarlato *et al*., 2001), turnover of RNA is expected to be a mechanism regulating gene expression in *H. pylori*. It would be interesting to investigate whether other *H. pylori* genes are regulated post-transcriptionally by mRNA decay and whether this is mediated by 5' stem loop structures.

7.3.2 VacA and disease

In Western populations such as the USA and Western Europe, strains of the *vacA* s1/m1 type have been shown to be associated with peptic ulceration, and gastric cancer (Atherton *et al*., 1995; Atherton *et al*., 1997; Rhead *et al*., 2007; Basso *et al*., 2008). The *vacA* i1 type is also a risk factor for peptic ulcer

disease, duodenal ulcer disease and gastric cancer (Basso *et al*., 2008; Rhead *et al*., 2007). In East Asian and Southeast Asian countries the association between *vacA* allelic diversity and clinical outcome is not well established as the population is predominantly infected with strains of the s1/i1 type (Maeda *et al*., 1998; Yamaoka *et al*., 1998). There is a high incidence of gastric cancer in this population which could be explained by the high prevalence of the more virulent *vacA* s1/i1 type strains (Rhead *et al*., 2007). However, not all individuals infected with more virulent strains go on to develop disease. It would be interesting to repeat the study carried out in this chapter on different populations to determine whether differences in *vacA* expression between *H. pylori* strains of the same *vacA* type *in vivo* could explain the imperfect correlation between *vacA* type and disease.

Studies have shown that the pattern of gastritis is important in determining disease outcome with antral predominant gastritis being associated with duodenal ulceration and a pangastritis being epidemiologically linked with gastric ulcers and gastric adenocarcinoma. It would be interesting to look at whether there is a correlation between the level of *vacA* transcript in the antrum and in the corpus, and to investigate whether there is a relationship between *vacA* transcript level and the pattern of gastritis.

The *vacA* genotype of an infecting strain has been found to be a good marker of disease. Work in this thesis showed that there is a relationship between *vacA* transcript levels produced by a strain and the severity of inflammation, activity and atrophy in the corpus. Several putative virulence factors including the *cag*

pathogenicity island, BabA and in-frame OipA alleles are present in strains of the more toxic s1 *vacA* type more frequently than in strains of the non-toxic *vacA* s2 type (Atherton *et al*., 1995; van Doorn *et al*., 1998; Gerhard *et al*., 1999; Cao and Cover, 2002; Yamaoka *et al*., 2002; Dossumbekova *et al*., 2006). It is therefore difficult to determine which of these putative virulence factors is the most important in the pathogenesis of gastroduodenal disease. However other factors such as variations in acid production, the immune response of the infected individual, environmental factors such as smoking and diet and other *H. pylori* virulence factors may also have an effect on histopathological severity and the disease status of the patient. It is likely that the development of disease in humans is not due to the action of one single factor and that a combination of these factors contribute to development of disease. It is important to carry out further studies to assess the relative importance of these other factors in *H. pylori* associated disease.

Chapter 8

8 References

ACHTMAN, M. & SUERBAUM, S. (2000) Sequence variation in Helicobacter pylori. *Trends Microbiol,* 8**,** 57-8.

AKADA, J. K., SHIRAI, M., TAKEUCHI, H., TSUDA, M. & NAKAZAWA, T. (2000) Identification of the urease operon in Helicobacter pylori and its control by mRNA decay in response to pH. *Mol Microbiol,* 36**,** 1071-84.

AKOPYANTS, N. S., CLIFTON, S. W., KERSULYTE, D., CRABTREE, J. E., YOUREE, B. E., REECE, C. A., BUKANOV, N. O., DRAZEK, E. S., ROE, B. A. & BERG, D. E. (1998) Analyses of the cag pathogenicity island of Helicobacter pylori. *Molecular Microbiology,* 28**,** 37-53.

ALBERTSON, N. H., NYSTROM, T. & KJELLEBERG, S. (1990) Exoprotease Activity of Two Marine Bacteria during Starvation. *Appl Environ Microbiol,* 56**,** 218-223.

ALGOOD, H. M., TORRES, V. J., UNUTMAZ, D. & COVER, T. L. (2007) Resistance of primary murine CD4+ T cells to Helicobacter pylori vacuolating cytotoxin. *Infection & Immunity,* 75**,** 334-41.

ALLAN, E., CLAYTON, C. L., MCLAREN, A., WALLACE, D. M. & WREN, B. W. (2001) Characterization of the low-pH responses of Helicobacter pylori using genomic DNA arrays. *Microbiology,* 147**,** 2285-92.

ALM, R. A., LING, L. S., MOIR, D. T., KING, B. L., BROWN, E. D., DOIG, P. C., SMITH, D. R., NOONAN, B., GUILD, B. C., DEJONGE, B. L., CARMEL, G., TUMMINO, P. J., CARUSO, A., URIA-NICKELSEN, M., MILLS, D. M., IVES, C., GIBSON, R., MERBERG, D., MILLS, S. D., JIANG, Q., TAYLOR, D. E., VOVIS, G. F. & TRUST, T. J. (1999) Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen Helicobacter pylori. *Nature,* 397**,** 176-80.

ANDRUTIS, K. A., FOX, J. G., SCHAUER, D. B., MARINI, R. P., MURPHY, J. C., YAN, L. & SOLNICK, J. V. (1995) Inability of an isogenic urease-negative mutant stain of Helicobacter mustelae to colonize the ferret stomach. *Infect Immun,* 63**,** 3722-5.

ANG, S., LEE, C. Z., PECK, K., SINDICI, M., MATRUBUTHAM, U., GLEESON, M. A. & WANG, J. T. (2001) Acid-induced gene expression in Helicobacter pylori: study in genomic scale by microarray. *Infect Immun,* 69**,** 1679-86.

ANTELMANN, H., TJALSMA, H., VOIGT, B., OHLMEIER, S., BRON, S., VAN DIJL, J. M. & HECKER, M. (2001) A proteomic view on genome-based signal peptide predictions. *Genome Res,* 11**,** 1484-502.

APPELMELK, B. J., NEGRINI, R., MORAN, A. P. & KUIPERS, E. J. (1997) Molecular mimicry between Helicobacter pylori and the host. *Trends Microbiol,* 5**,** 70-3.

ARENTS, N. L., VAN ZWET, A. A., THIJS, J. C., KOOISTRA-SMID, A. M., VAN SLOCHTEREN, K. R., DEGENER, J. E., KLEIBEUKER, J. H. & VAN DOORN, L. J. (2001) The importance of vacA, cagA, and iceA genotypes of Helicobacter pylori infection in peptic ulcer disease and gastroesophageal reflux disease. *Am J Gastroenterol,* 96**,** 2603-8.

ARGENT, R. H., BURETTE, A., MIENDJE DEYI, V. Y. & ATHERTON, J. C. (2007) The presence of dupA in Helicobacter pylori is not significantly associated with duodenal ulceration in Belgium, South Africa, China, or North America. *Clin Infect Dis,* 45**,** 1204-6.

ATHERTON, J. C. (2006) The pathogenesis of Helicobacter pylori-induced gastro-duodenal diseases. *Annu Rev Pathol,* 1**,** 63-96.

ATHERTON, J. C., CAO, P., PEEK, R. M., JR., TUMMURU, M. K., BLASER, M. J. & COVER, T. L. (1995) Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. *Journal of Biological Chemistry,* 270**,** 17771-7.

ATHERTON, J. C., PEEK, R. M., JR., THAM, K. T., COVER, T. L. & BLASER, M. J. (1997) Clinical and pathological importance of heterogeneity in vacA, the vacuolating cytotoxin gene of Helicobacter pylori. *Gastroenterology,* 112**,** 92-9.

AUDIA, J. P., WEBB, C. C. & FOSTER, J. W. (2001) Breaking through the acid barrier: an orchestrated response to proton stress by enteric bacteria. *Int J Med Microbiol,* 291**,** 97-106.

AYALA, G., CHIHU, L., PERALES, G., FIERROS-ZARATE, G., HANSEN, L. M., SOLNICK, J. V. & SANCHEZ, J. (2004) Quantitation of H. pylori cytotoxin mRNA by real-time RT-PCR shows a wide expression range that does not correlate with promoter sequences. *Microb Pathog,* 37**,** 163-7.

AZUMA, T., YAMAKAWA, A., YAMAZAKI, S., FUKUTA, K., OHTANI, M., ITO, Y., DOJO, M., YAMAZAKI, Y. & KURIYAMA, M. (2002) Correlation between variation of the 3' region of the cagA gene in Helicobacter pylori and disease outcome in Japan. *J Infect Dis,* 186**,** 1621-30.

BACH, S., MAKRISTATHIS, A., ROTTER, M. & HIRSCHL, A. M. (2002) Gene expression profiling in AGS cells stimulated with Helicobacter pylori isogenic strains (cagA positive or cagA negative). *Infect Immun,* 70**,** 988-92.

BACKHED, F., ROKBI, B., TORSTENSSON, E., ZHAO, Y., NILSSON, C., SEGUIN, D., NORMARK, S., BUCHAN, A. M. & RICHTER-DAHLFORS, A. (2003) Gastric mucosal recognition of Helicobacter pylori is independent of Toll-like receptor 4. *J Infect Dis,* 187**,** 829-36.

BAMFORD, K. B., FAN, X., CROWE, S. E., LEARY, J. F., GOURLEY, W. K., LUTHRA, G. K., BROOKS, E. G., GRAHAM, D. Y., REYES, V. E. & ERNST, P. B. (1998) Lymphocytes in the human gastric mucosa during Helicobacter pylori have a T helper cell 1 phenotype. *Gastroenterology,* 114**,** 482-92.

BANDYOPADHYAY, D., BISWAS, K., BHATTACHARYYA, M., REITER, R. J. & BANERJEE, R. K. (2001) Gastric toxicity and mucosal

ulceration induced by oxygen-derived reactive species: protection by melatonin. *Curr Mol Med,* 1**,** 501-13.

BARKOCY-GALLAGHER, G. A. & BASSFORD, P. J., JR. (1992) Synthesis of precursor maltose-binding protein with proline in the +1 position of the cleavage site interferes with the activity of Escherichia coli signal peptidase I in vivo. *J Biol Chem,* 267**,** 1231-8.

BARNARD, F. M., LOUGHLIN, M. F., FAINBERG, H. P., MESSENGER, M. P., USSERY, D. W., WILLIAMS, P. & JENKS, P. J. (2004) Global regulation of virulence and the stress response by CsrA in the highly adapted human gastric pathogen Helicobacter pylori. *Mol Microbiol,* 51**,** 15-32.

BASSO, D., ZAMBON, C. F., LETLEY, D. P., STRANGES, A., MARCHET, A., RHEAD, J. L., SCHIAVON, S., GUARISO, G., CEROTI, M., NITTI, D., RUGGE, M., PLEBANI, M. & ATHERTON, J. C. (2008) Clinical relevance of Helicobacter pylori cagA and vacA gene polymorphisms. *Gastroenterology,* 135**,** 91-9.

BEALES, I. L., CRABTREE, J. E., SCUNES, D., COVACCI, A. & CALAM, J. (1996) Antibodies to CagA protein are associated with gastric atrophy in Helicobacter pylori infection. *Eur J Gastroenterol Hepatol,* 8**,** 645-9.

BECHHOFER, D. H. & DUBNAU, D. (1987) Induced mRNA stability in Bacillus subtilis. *Proc Natl Acad Sci U S A,* 84**,** 498-502.

BEIER, D., SPOHN, G., RAPPUOLI, R. & SCARLATO, V. (1997) Identification and characterization of an operon of Helicobacter pylori that is involved in motility and stress adaptation. *J Bacteriol,* 179**,** 4676-83.

BEIER, D., SPOHN, G., RAPPUOLI, R. & SCARLATO, V. (1998) Functional analysis of the Helicobacter pylori principal sigma subunit of RNA polymerase reveals that the spacer region is important for efficient transcription. *Mol Microbiol,* 30**,** 121-34.

BELASCO, J. G., NILSSON, G., VON GABAIN, A. & COHEN, S. N. (1986) The stability of E. coli gene transcripts is dependent on determinants localized to specific mRNA segments. *Cell,* 46**,** 245-51.

BERESWILL, S., FASSBINDER, F., VOLZING, C., COVACCI, A., HAAS, R. & KIST, M. (1998) Hemolytic properties and riboflavin synthesis of Helicobacter pylori: cloning and functional characterization of the ribA gene encoding GTP-cyclohydrolase II that confers hemolytic activity to Escherichia coli. *Medical Microbiology & Immunology,* 186**,** 177-87.

BERESWILL, S., GREINER, S., VAN VLIET, A. H., WAIDNER, B., FASSBINDER, F., SCHILTZ, E., KUSTERS, J. G. & KIST, M. (2000) Regulation of ferritin-mediated cytoplasmic iron storage by the ferric uptake regulator homolog (Fur) of Helicobacter pylori. *J Bacteriol,* 182**,** 5948-53.

BIJLSMA, J. J., WAIDNER, B., VLIET, A. H., HUGHES, N. J., HAG, S., BERESWILL, S., KELLY, D. J., VANDENBROUCKE-GRAULS, C. M., KIST, M. & KUSTERS, J. G. (2002) The Helicobacter pylori homologue of the ferric uptake regulator is involved in acid resistance. *Infect Immun,* 70**,** 606-11.

BLASER, M. J. (1995) Intrastrain differences in Helicobacter pylori: a key question in mucosal damage? *Annals of Medicine,* 27**,** 559-63.

BONCRISTIANO, M., PACCANI, S. R., BARONE, S., ULIVIERI, C., PATRUSSI, L., ILVER, D., AMEDEI, A., D'ELIOS, M. M., TELFORD, J. L. & BALDARI, C. T. (2003) The Helicobacter pylori vacuolating toxin inhibits T cell activation by two independent mechanisms. *J Exp Med,* 198**,** 1887-97.

BOONJAKUAKUL, J. K., CANFIELD, D. R. & SOLNICK, J. V. (2005) Comparison of Helicobacter pylori virulence gene expression in vitro and in the Rhesus macaque. *Infect Immun,* 73**,** 4895-904.

BOONJAKUAKUL, J. K., SYVANEN, M., SURYAPRASAD, A., BOWLUS, C. L. & SOLNICK, J. V. (2004) Transcription profile of Helicobacter pylori in the human stomach reflects its physiology in vivo. *J Infect Dis,* 190**,** 946-56.

BOUGHAN, P. K., ARGENT, R. H., BODY-MALAPEL, M., PARK, J. H., EWINGS, K. E., BOWIE, A. G., ONG, S. J., COOK, S. J., SORENSEN, O. E., MANZO, B. A., INOHARA, N., KLEIN, N. J., NUNEZ, G., ATHERTON, J. C. & BAJAJ-ELLIOTT, M. (2006) Nucleotide-binding oligomerization domain-1 and epidermal growth factor receptor: critical regulators of beta-defensins during Helicobacter pylori infection. *J Biol Chem,* 281**,** 11637-48.

BOURZAC, K. M. & GUILLEMIN, K. (2005) Helicobacter pylori-host cell interactions mediated by type IV secretion. *Cell Microbiol,* 7**,** 911-9.

BOUVET, P. & BELASCO, J. G. (1992) Control of RNase E-mediated RNA degradation by 5'-terminal base pairing in E. coli. *Nature,* 360**,** 488-91.

BRANDI, A., PIETRONI, P., GUALERZI, C. O. & PON, C. L. (1996) Post-transcriptional regulation of CspA expression in Escherichia coli. *Mol Microbiol,* 19**,** 231-40.

BRAWERMAN, G. (1987) Determinants of messenger RNA stability. *Cell,* 48**,** 5-6.

BREST, P., HOFMAN, V., LASSALLE, S., CESARO, A., RICCI, V., SELVA, E., AUBERGER, P. & HOFMAN, P. (2006) Human polymorphonuclear leukocytes are sensitive in vitro to Helicobacter pylori vaca toxin. *Helicobacter,* 11**,** 544-55.

BRICKER, A. L. & BELASCO, J. G. (1999) Importance of a 5' stem-loop for longevity of papA mRNA in Escherichia coli. *J Bacteriol,* 181**,** 3587-90.

BUCCA, G., FERINA, G., PUGLIA, A. M. & SMITH, C. P. (1995) The dnaK operon of Streptomyces coelicolor encodes a novel heat-shock protein which binds to the promoter region of the operon. *Mol Microbiol,* 17**,** 663-74.

BUCCA, G., HINDLE, Z. & SMITH, C. P. (1997) Regulation of the dnaK operon of Streptomyces coelicolor A3(2) is governed by HspR, an autoregulatory repressor protein. *J Bacteriol,* 179**,** 5999-6004.

BURNS, B. P., HAZELL, S. L. & MENDZ, G. L. (1995) Acetyl-CoA carboxylase activity in Helicobacter pylori and the requirement of increased CO2 for growth. *Microbiology,* 141 (Pt 12)**,** 3113-8.

BURNS, B. P., HAZELL, S. L., MENDZ, G. L., KOLESNIKOW, T., TILLET, D. & NEILAN, B. A. (2000) The Helicobacter pylori pyrB

gene encoding aspartate carbamoyltransferase is essential for bacterial survival. *Arch Biochem Biophys,* 380**,** 78-84.

BURY-MONE, S., THIBERGE, J. M., CONTRERAS, M., MAITOURNAM, A., LABIGNE, A. & DE REUSE, H. (2004) Responsiveness to acidity via metal ion regulators mediates virulence in the gastric pathogen Helicobacter pylori. *Mol Microbiol,* 53**,** 623-38.

BUSTIN, S. A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol,* 25**,** 169-93.

CANNISTRARO, V. J., SUBBARAO, M. N. & KENNELL, D. (1986) Specific endonucleolytic cleavage sites for decay of Escherichia coli mRNA. *J Mol Biol,* 192**,** 257-74.

CARRIER, T. A. & KEASLING, J. D. (1997a) Controlling messenger RNA stability in bacteria: strategies for engineering gene expression. *Biotechnol Prog,* 13**,** 699-708.

CARRIER, T. A. & KEASLING, J. D. (1997b) Mechanistic modeling of prokaryotic mRNA decay. *J Theor Biol,* 189**,** 195-209.

CENSINI, S., LANGE, C., XIANG, Z., CRABTREE, J. E., GHIARA, P., BORODOVSKY, M., RAPPUOLI, R. & COVACCI, A. (1996) cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci U S A,* 93**,** 14648-53.

CHOI, K. M., LIM, W. J., PARK, J. K. & HWANG, S. Y. (2001) Presumptive mechanisms of peptic ulceration by Helicobacter pylori VacA involving mucoprotease and CagA. *Mol Cells,* 11**,** 312-20.

CONDON, C., PUTZER, H., LUO, D. & GRUNBERG-MANAGO, M. (1997) Processing of the Bacillus subtilis thrS leader mRNA is RNase E-dependent in Escherichia coli. *J Mol Biol,* 268**,** 235-42.

CONTRERAS, M., THIBERGE, J. M., MANDRAND-BERTHELOT, M. A. & LABIGNE, A. (2003) Characterization of the roles of NikR, a nickel-responsive pleiotropic autoregulator of Helicobacter pylori. *Mol Microbiol,* 49**,** 947-63.

CORREA, P. (1996) Helicobacter pylori and gastric cancer: state of the art. *Cancer Epidemiology, Biomarkers & Prevention,* 5**,** 477-81.

CORREA, P., HAENSZEL, W., CUELLO, C., TANNENBAUM, S. & ARCHER, M. (1975) A model for gastric cancer epidemiology. *Lancet,* 2**,** 58-60.

COVER, T. L., CAO, P., LIND, C. D., THAM, K. T. & BLASER, M. J. (1993) Correlation between vacuolating cytotoxin production by Helicobacter pylori isolates in vitro and in vivo. *Infection & Immunity,* 61**,** 5008-12.

COVER, T. L., CAO, P., MURTHY, U. K., SIPPLE, M. S. & BLASER, M. J. (1992) Serum neutralizing antibody response to the vacuolating cytotoxin of Helicobacter pylori. *Journal of Clinical Investigation,* 90**,** 913-8.

COVER, T. L., HANSON, P. I. & HEUSER, J. E. (1997) Acid-induced dissociation of VacA, the Helicobacter pylori vacuolating cytotoxin, reveals its pattern of assembly. *Journal of Cell Biology,* 138**,** 759-69.

COVER, T. L., TUMMURU, M. K., CAO, P., THOMPSON, S. A. & BLASER, M. J. (1994) Divergence of genetic sequences for the vacuolating cytotoxin among Helicobacter pylori strains. *J Biol Chem,* 269**,** 10566-73.

CRABTREE, J. E., PEICHL, P., WYATT, J. I., STACHL, U. & LINDLEY, I. J. (1993) Gastric interleukin-8 and IgA IL-8 autoantibodies in Helicobacter pylori infection. *Scand J Immunol,* 37**,** 65-70.

CRABTREE, J. E., SHALLCROSS, T. M., HEATLEY, R. V. & WYATT, J. I. (1991) Mucosal tumour necrosis factor alpha and interleukin-6 in patients with Helicobacter pylori associated gastritis. *Gut,* 32**,** 1473-7.

CZAJKOWSKY, D. M., IWAMOTO, H., COVER, T. L. & SHAO, Z. (1999) The vacuolating toxin from Helicobacter pylori forms hexameric pores in lipid bilayers at low pH. *Proc Natl Acad Sci U S A,* 96**,** 2001-6.

DALBEY, R. E. & WICKNER, W. (1985) Leader peptidase catalyzes the release of exported proteins from the outer surface of the Escherichia coli plasma membrane. *J Biol Chem,* 260**,** 15925-31.

DE BERNARD, M., CAPPON, A., PANCOTTO, L., RUGGIERO, P., RIVERA, J., DEL GIUDICE, G. & MONTECUCCO, C. (2005) The Helicobacter pylori VacA cytotoxin activates RBL-2H3 cells by inducing cytosolic calcium oscillations. *Cellular Microbiology,* 7**,** 191-8.

DE BERNARD, M., MOSCHIONI, M., NAPOLITANI, G., RAPPUOLI, R. & MONTECUCCO, C. (2000) The VacA toxin of Helicobacter pylori identifies a new intermediate filament-interacting protein. *EMBO J,* 19**,** 48-56.

DE VRIES, N., KUIPERS, E. J., KRAMER, N. E., VAN VLIET, A. H., BIJLSMA, J. J., KIST, M., BERESWILL, S., VANDENBROUCKE-GRAULS, C. M. & KUSTERS, J. G. (2001) Identification of environmental stress-regulated genes in Helicobacter pylori by a lacZ reporter gene fusion system. *Helicobacter,* 6**,** 300-9.

DELANY, I., PACHECO, A. B., SPOHN, G., RAPPUOLI, R. & SCARLATO, V. (2001a) Iron-dependent transcription of the frpB gene of Helicobacter pylori is controlled by the Fur repressor protein. *J Bacteriol,* 183**,** 4932-7.

DELANY, I., SPOHN, G., RAPPUOLI, R. & SCARLATO, V. (2001b) The Fur repressor controls transcription of iron-activated and -repressed genes in Helicobacter pylori. *Mol Microbiol,* 42**,** 1297-309.

DELANY, I., SPOHN, G., RAPPUOLI, R. & SCARLATO, V. (2002a) Growth phase-dependent regulation of target gene promoters for binding of the essential orphan response regulator HP1043 of Helicobacter pylori. *J Bacteriol,* 184**,** 4800-10.

DELANY, I., SPOHN, G., RAPPUOLI, R. & SCARLATO, V. (2002b) In vitro selection of high affinity HspR-binding sites within the genome of Helicobacter pylori. *Gene,* 283**,** 63-9.

DING, S. Z., TOROK, A. M., SMITH, M. F., JR. & GOLDBERG, J. B. (2005) Toll-like receptor 2-mediated gene expression in epithelial cells during Helicobacter pylori infection. *Helicobacter,* 10**,** 193-204.

DIWA, A., BRICKER, A. L., JAIN, C. & BELASCO, J. G. (2000) An evolutionarily conserved RNA stem-loop functions as a sensor that

directs feedback regulation of RNase E gene expression. *Genes Dev,* 14**,** 1249-60.

DIXON, M. F., GENTA, R. M., YARDLEY, J. H. & CORREA, P. (1996) Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol,* 20**,** 1161-81.

DOSSUMBEKOVA, A., PRINZ, C., GERHARD, M., BRENNER, L., BACKERT, S., KUSTERS, J. G., SCHMID, R. M. & RAD, R. (2006) Helicobacter pylori outer membrane proteins and gastric inflammation. *Gut,* 55**,** 1360-1; author reply 1361.

DUNDON, W. G., MARSHALL, D. G., MORAIN, C. A. & SMYTH, C. J. (1999) A novel tRNA-associated locus (trl) from Helicobacter pylori is co-transcribed with tRNA(Gly) and reveals genetic diversity. *Microbiology,* 145 (Pt 6)**,** 1289-98.

EATON, K. A. & KRAKOWKA, S. (1994) Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by Helicobacter pylori. *Infect Immun,* 62**,** 3604-7.

EATON, K. A., SUERBAUM, S., JOSENHANS, C. & KRAKOWKA, S. (1996) Colonization of gnotobiotic piglets by Helicobacter pylori deficient in two flagellin genes. *Infect Immun,* 64**,** 2445-8.

EHRETSMANN, C. P., CARPOUSIS, A. J. & KRISCH, H. M. (1992a) mRNA degradation in procaryotes. *FASEB J,* 6**,** 3186-92.

EHRETSMANN, C. P., CARPOUSIS, A. J. & KRISCH, H. M. (1992b) Specificity of Escherichia coli endoribonuclease RNase E: in vivo and in vitro analysis of mutants in a bacteriophage T4 mRNA processing site. *Genes Dev,* 6**,** 149-59.

EL-OMAR, E. M., CARRINGTON, M., CHOW, W. H., MCCOLL, K. E., BREAM, J. H., YOUNG, H. A., HERRERA, J., LISSOWSKA, J., YUAN, C. C., ROTHMAN, N., LANYON, G., MARTIN, M., FRAUMENI, J. F., JR. & RABKIN, C. S. (2000) Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature,* 404**,** 398-402.

EMORY, S. A. & BELASCO, J. G. (1990) The ompA 5' untranslated RNA segment functions in Escherichia coli as a growth-rate-regulated mRNA stabilizer whose activity is unrelated to translational efficiency. *J Bacteriol,* 172**,** 4472-81.

EMORY, S. A., BOUVET, P. & BELASCO, J. G. (1992) A 5'-terminal stem-loop structure can stabilize mRNA in Escherichia coli. *Genes Dev,* 6**,** 135-48.

ENROTH, H., WREIBER, K., RIGO, R., RISBERG, D., URIBE, A. & ENGSTRAND, L. (1999) In vitro aging of Helicobacter pylori: changes in morphology, intracellular composition and surface properties. *Helicobacter,* 4**,** 7-16.

ERMAK, T. H., GIANNASCA, P. J., NICHOLS, R., MYERS, G. A., NEDRUD, J., WELTZIN, R., LEE, C. K., KLEANTHOUS, H. & MONATH, T. P. (1998) Immunization of mice with urease vaccine affords protection against Helicobacter pylori infection in the absence of antibodies and is mediated by MHC class II-restricted responses. *J Exp Med,* 188**,** 2277-88.

ERNST, F. D., BERESWILL, S., WAIDNER, B., STOOF, J., MADER, U., KUSTERS, J. G., KUIPERS, E. J., KIST, M., VAN VLIET, A. H. & HOMUTH, G. (2005a) Transcriptional profiling of Helicobacter pylori Fur- and iron-regulated gene expression. *Microbiology,* 151**,** 533-46.

ERNST, F. D., KUIPERS, E. J., HEIJENS, A., SARWARI, R., STOOF, J., PENN, C. W., KUSTERS, J. G. & VAN VLIET, A. H. (2005b) The nickel-responsive regulator NikR controls activation and repression of gene transcription in Helicobacter pylori. *Infect Immun,* 73**,** 7252-8.

ERNST, P. B. & GOLD, B. D. (2000) The disease spectrum of Helicobacter pylori: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. *Annu Rev Microbiol,* 54**,** 615-40.

EVANS, D. G., QUEIROZ, D. M., MENDES, E. N. & EVANS, D. J., JR. (1998) Helicobacter pylori cagA status and s and m alleles of vacA in isolates from individuals with a variety of H. pylori-associated gastric diseases. *J Clin Microbiol,* 36**,** 3435-7.

FALK, G. W. (1995) Current status of Helicobacter pylori in peptic ulcer disease. *Cleve Clin J Med,* 62**,** 95-104.

FALUSH, D., KRAFT, C., TAYLOR, N. S., CORREA, P., FOX, J. G., ACHTMAN, M. & SUERBAUM, S. (2001) Recombination and mutation during long-term gastric colonization by Helicobacter pylori: estimates of clock rates, recombination size, and minimal age. *Proc Natl Acad Sci U S A,* 98**,** 15056-61.

FAN, X. J., CHUA, A., SHAHI, C. N., MCDEVITT, J., KEELING, P. W. & KELLEHER, D. (1994) Gastric T lymphocyte responses to Helicobacter pylori in patients with H pylori colonisation. *Gut,* 35**,** 1379-84.

FANG, L., JIANG, W., BAE, W. & INOUYE, M. (1997) Promoter-independent cold-shock induction of cspA and its derepression at 37 degrees C by mRNA stabilization. *Mol Microbiol,* 23**,** 355-64.

FERRARI, G., LANGEN, H., NAITO, M. & PIETERS, J. (1999) A coat protein on phagosomes involved in the intracellular survival of mycobacteria. *Cell,* 97**,** 435-47.

FERRERO, R. L. (2005) Innate immune recognition of the extracellular mucosal pathogen, Helicobacter pylori. *Mol Immunol,* 42**,** 879-85.

FIKES, J. D., BARKOCY-GALLAGHER, G. A., KLAPPER, D. G. & BASSFORD, P. J., JR. (1990) Maturation of Escherichia coli maltose-binding protein by signal peptidase I in vivo. Sequence requirements for efficient processing and demonstration of an alternate cleavage site. *J Biol Chem,* 265**,** 3417-23.

FIOCCA, R., NECCHI, V., SOMMI, P., RICCI, V., TELFORD, J., COVER, T. L. & SOLCIA, E. (1999) Release of Helicobacter pylori vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium. *Journal of Pathology,* 188**,** 220-6.

FITCHEN, N., LETLEY, D. P., O'SHEA, P., ATHERTON, J. C., WILLIAMS, P. & HARDIE, K. R. (2005) All subtypes of the cytotoxin VacA adsorb to the surface of Helicobacter pylori post-secretion. *Journal of Medical Microbiology,* 54**,** 621-30.

FORSYTH, M. H., ATHERTON, J. C., BLASER, M. J. & COVER, T. L. (1998) Heterogeneity in levels of vacuolating cytotoxin gene (vacA) transcription among Helicobacter pylori strains. *Infection & Immunity,* 66**,** 3088-94.

FORSYTH, M. H. & COVER, T. L. (1999) Mutational analysis of the vacA promoter provides insight into gene transcription in Helicobacter pylori. *J Bacteriol,* 181**,** 2261-6.

FORSYTH, M. H. & COVER, T. L. (2000) Intercellular communication in Helicobacter pylori: luxS is essential for the production of an extracellular signaling molecule. *Infect Immun,* 68**,** 3193-9.

FREEMAN, W. M., WALKER, S. J. & VRANA, K. E. (1999) Quantitative RT-PCR: pitfalls and potential. *Biotechniques,* 26**,** 112-22, 124-5.

FRONHOFFS, S., TOTZKE, G., STIER, S., WERNERT, N., ROTHE, M., BRUNING, T., KOCH, B., SACHINIDIS, A., VETTER, H. & KO, Y. (2002) A method for the rapid construction of cRNA standard curves in quantitative real-time reverse transcription polymerase chain reaction. *Mol Cell Probes,* 16**,** 99-110.

GALMICHE, A., RASSOW, J., DOYE, A., CAGNOL, S., CHAMBARD, J. C., CONTAMIN, S., DE THILLOT, V., JUST, I., RICCI, V., SOLCIA, E., VAN OBBERGHEN, E. & BOQUET, P. (2000) The N-terminal 34 kDa fragment of Helicobacter pylori vacuolating cytotoxin targets mitochondria and induces cytochrome c release. *EMBO Journal,* 19**,** 6361-70.

GANCZ, H., CENSINI, S. & MERRELL, D. S. (2006) Iron and pH homeostasis intersect at the level of Fur regulation in the gastric pathogen Helicobacter pylori. *Infect Immun,* 74**,** 602-14.

GARHART, C. A., REDLINE, R. W., NEDRUD, J. G. & CZINN, S. J. (2002) Clearance of Helicobacter pylori Infection and Resolution of Postimmunization Gastritis in a Kinetic Study of Prophylactically Immunized Mice. *Infect Immun,* 70**,** 3529-38.

GARNER, J. A. & COVER, T. L. (1995) Analysis of genetic diversity in cytotoxin-producing and non-cytotoxin-producing Helicobacter pylori strains. *J Infect Dis,* 172**,** 290-3.

GEBERT, B., FISCHER, W. & HAAS, R. (2004) The Helicobacter pylori vacuolating cytotoxin: from cellular vacuolation to immunosuppressive activities. *Reviews of Physiology Biochemistry & Pharmacology,* 152**,** 205-20.

GEBERT, B., FISCHER, W., WEISS, E., HOFFMANN, R. & HAAS, R. (2003) Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. *Science,* 301**,** 1099-102.

GEORGE, J. T., BOUGHAN, P. K., KARAGEORGIOU, H. & BAJAJ-ELLIOTT, M. (2003) Host anti-microbial response to Helicobacter pylori infection. *Mol Immunol,* 40**,** 451-6.

GEORGELLIS, D., ARVIDSON, S. & VON GABAIN, A. (1992) Decay of ompA mRNA and processing of 9S RNA are immediately affected by shifts in growth rate, but in opposite manners. *J Bacteriol,* 174**,** 5382-90.

GEORGELLIS, D., BARLOW, T., ARVIDSON, S. & VON GABAIN, A. (1993) Retarded RNA turnover in Escherichia coli: a means of

maintaining gene expression during anaerobiosis. *Mol Microbiol,* 9**,** 375-81.

GEWIRTZ, A. T., YU, Y., KRISHNA, U. S., ISRAEL, D. A., LYONS, S. L. & PEEK, R. M., JR. (2004) Helicobacter pylori flagellin evades toll-like receptor 5-mediated innate immunity. *J Infect Dis,* 189**,** 1914-20.

GHIARA, P., MARCHETTI, M., BLASER, M. J., TUMMURU, M. K., COVER, T. L., SEGAL, E. D., TOMPKINS, L. S. & RAPPUOLI, R. (1995) Role of the Helicobacter pylori virulence factors vacuolating cytotoxin, CagA, and urease in a mouse model of disease. *Infection & Immunity,* 63**,** 4154-60.

GIESELER, S., KONIG, B., KONIG, W. & BACKERT, S. (2005) Strain-specific expression profiles of virulence genes in Helicobacter pylori during infection of gastric epithelial cells and granulocytes. *Microbes Infect,* 7**,** 437-47.

GOLDENBERG, D., AZAR, I. & OPPENHEIM, A. B. (1996) Differential mRNA stability of the cspA gene in the cold-shock response of Escherichia coli. *Mol Microbiol,* 19**,** 241-8.

GOODWIN, C. S., BLINCOW, E. D., WARREN, J. R., WATERS, T. E., SANDERSON, C. R. & EASTON, L. (1985) Evaluation of cultural techniques for isolating Campylobacter pyloridis from endoscopic biopsies of gastric mucosa. *J Clin Pathol,* 38**,** 1127-31.

GRAHAM, J. E., PEEK, R. M., JR., KRISHNA, U. & COVER, T. L. (2002) Global analysis of Helicobacter pylori gene expression in human gastric mucosa. *Gastroenterology,* 123**,** 1637-48.

GRANDVALET, C., DE CRECY-LAGARD, V. & MAZODIER, P. (1999) The ClpB ATPase of Streptomyces albus G belongs to the HspR heat shock regulon. *Mol Microbiol,* 31**,** 521-32.

HAMBRAEUS, G., PERSSON, M. & RUTBERG, B. (2000) The aprE leader is a determinant of extreme mRNA stability in Bacillus subtilis. *Microbiology,* 146 Pt 12**,** 3051-9.

HANSEN, M. J., CHEN, L. H., FEJZO, M. L. & BELASCO, J. G. (1994) The ompA 5' untranslated region impedes a major pathway for mRNA degradation in Escherichia coli. *Mol Microbiol,* 12**,** 707-16.

HENDERSON, I. R., NAVARRO-GARCIA, F. & NATARO, J. P. (1998) The great escape: structure and function of the autotransporter proteins. *Trends Microbiol,* 6**,** 370-8.

HENDERSON, I. R. & OWEN, P. (1999) The major phase-variable outer membrane protein of Escherichia coli structurally resembles the immunoglobulin A1 protease class of exported protein and is regulated by a novel mechanism involving Dam and oxyR. *J Bacteriol,* 181**,** 2132-41.

HENGGE-ARONIS, R. (2002a) Recent insights into the general stress response regulatory network in Escherichia coli. *J Mol Microbiol Biotechnol,* 4**,** 341-6.

HENGGE-ARONIS, R. (2002b) Stationary phase gene regulation: what makes an Escherichia coli promoter sigmaS-selective? *Curr Opin Microbiol,* 5**,** 591-5.

HESTERKAMP, T. & BUKAU, B. (1998) Role of the DnaK and HscA homologs of Hsp70 chaperones in protein folding in E.coli. *EMBO J,* 17**,** 4818-28.

HEUERMANN, D. & HAAS, R. (1995) Genetic organization of a small cryptic plasmid of Helicobacter pylori. *Gene,* 165**,** 17-24.

HIGASHI, H., NAKAYA, A., TSUTSUMI, R., YOKOYAMA, K., FUJII, Y., ISHIKAWA, S., HIGUCHI, M., TAKAHASHI, A., KURASHIMA, Y., TEISHIKATA, Y., TANAKA, S., AZUMA, T. & HATAKEYAMA, M. (2004) Helicobacter pylori CagA induces Ras-independent morphogenetic response through SHP-2 recruitment and activation. *J Biol Chem,* 279**,** 17205-16.

HIRATA, Y., OHMAE, T., SHIBATA, W., MAEDA, S., OGURA, K., YOSHIDA, H., KAWABE, T. & OMATA, M. (2006) MyD88 and TNF receptor-associated factor 6 are critical signal transducers in Helicobacter pylori-infected human epithelial cells. *J Immunol,* 176**,** 3796-803.

HISATSUNE, J., YAMASAKI, E., NAKAYAMA, M., SHIRASAKA, D., KURAZONO, H., KATAGATA, Y., INOUE, H., HAN, J., SAP, J., YAHIRO, K., MOSS, J. & HIRAYAMA, T. (2007) Helicobacter pylori VacA enhances prostaglandin E2 production through induction of cyclooxygenase 2 expression via a p38 mitogen-activated protein kinase/activating transcription factor 2 cascade in AZ-521 cells. *Infect Immun,* 75**,** 4472-81.

HOMUTH, G., DOMM, S., KLEINER, D. & SCHUMANN, W. (2000) Transcriptional analysis of major heat shock genes of Helicobacter pylori. *J Bacteriol,* 182**,** 4257-63.

HORNEF, M. W. & BOGDAN, C. (2005) The role of epithelial Toll-like receptor expression in host defense and microbial tolerance. *J Endotoxin Res,* 11**,** 124-8.

HOTCHIN, N. A., COVER, T. L. & AKHTAR, N. (2000) Cell vacuolation induced by the VacA cytotoxin of Helicobacter pylori is regulated by the Rac1 GTPase. *J Biol Chem,* 275**,** 14009-12.

ILVER, D., BARONE, S., MERCATI, D., LUPETTI, P. & TELFORD, J. L. (2004) Helicobacter pylori toxin VacA is transferred to host cells via a novel contact-dependent mechanism. *Cell Microbiol,* 6**,** 167-74.

INUI, T., MIZUNO, S., TAKAI, K., NAKAGAWA, M., UCHIDA, M., FUJIMIYA, M., ASAKAWA, A. & INUI, A. (2003) Helicobacter pylori cytotoxin: a novel ligand for receptor-like protein tyrosine phosphatase beta (review). *International Journal of Molecular Medicine,* 12**,** 917-21.

ISHIHARA, S., RUMI, M. A., KADOWAKI, Y., ORTEGA-CAVA, C. F., YUKI, T., YOSHINO, N., MIYAOKA, Y., KAZUMORI, H., ISHIMURA, N., AMANO, Y. & KINOSHITA, Y. (2004) Essential role of MD-2 in TLR4-dependent signaling during Helicobacter pylori-associated gastritis. *J Immunol,* 173**,** 1406-16.

ISRAEL, D. A., SALAMA, N., ARNOLD, C. N., MOSS, S. F., ANDO, T., WIRTH, H. P., THAM, K. T., CAMORLINGA, M., BLASER, M. J., FALKOW, S. & PEEK, R. M., JR. (2001) Helicobacter pylori strain-

specific differences in genetic content, identified by microarray, influence host inflammatory responses. *J Clin Invest,* 107**,** 611-20.

IWAMOTO, H., CZAJKOWSKY, D. M., COVER, T. L., SZABO, G. & SHAO, Z. (1999) VacA from Helicobacter pylori: a hexameric chloride channel. *FEBS Lett,* 450**,** 101-4.

IZARD, J. W. & KENDALL, D. A. (1994) Signal peptides: exquisitely designed transport promoters. *Mol Microbiol,* 13**,** 765-73.

JI, X., FERNANDEZ, T., BURRONI, D., PAGLIACCIA, C., ATHERTON, J. C., REYRAT, J. M., RAPPUOLI, R. & TELFORD, J. L. (2000) Cell specificity of Helicobacter pylori cytotoxin is determined by a short region in the polymorphic midregion. *Infection & Immunity,* 68**,** 3754-7.

JIANG, X. & DOYLE, M. P. (2000) Growth supplements for Helicobacter pylori. *J Clin Microbiol,* 38**,** 1984-7.

JOSEPH, B. & BEIER, D. (2007) Global analysis of two-component gene regulation in H. pylori by mutation analysis and transcriptional profiling. *Methods Enzymol,* 423**,** 514-30.

JUNG, H. C., KIM, J. M., SONG, I. S. & KIM, C. Y. (1997) Helicobacter pylori induces an array of pro-inflammatory cytokines in human gastric epithelial cells: quantification of mRNA for interleukin-8, -1 alpha/beta, granulocyte-macrophage colony-stimulating factor, monocyte chemoattractant protein-1 and tumour necrosis factor-alpha. *J Gastroenterol Hepatol,* 12**,** 473-80.

KANSAU, I., RAYMOND, J., BINGEN, E., COURCOUX, P., KALACH, N., BERGERET, M., BRAIMI, N., DUPONT, C. & LABIGNE, A. (1996) Genotyping of Helicobacter pylori isolates by sequencing of PCR products and comparison with the RAPD technique. *Res Microbiol,* 147**,** 661-9.

KARAMYSHEV, A. L., KARAMYSHEVA, Z. N., KAJAVA, A. V., KSENZENKO, V. N. & NESMEYANOVA, M. A. (1998) Processing of Escherichia coli alkaline phosphatase: role of the primary structure of the signal peptide cleavage region. *J Mol Biol,* 277**,** 859-70.

KARIN, M. (2006) Nuclear factor-kappaB in cancer development and progression. *Nature,* 441**,** 431-6.

KEENAN, J., DAY, T., NEAL, S., COOK, B., PEREZ-PEREZ, G., ALLARDYCE, R. & BAGSHAW, P. (2000) A role for the bacterial outer membrane in the pathogenesis of Helicobacter pylori infection. *FEMS Microbiol Lett,* 182**,** 259-64.

KEENAN, J. I. & ALLARDYCE, R. A. (2000) Iron influences the expression of Helicobacter pylori outer membrane vesicle-associated virulence factors. *Eur J Gastroenterol Hepatol,* 12**,** 1267-73.

KIDD, M., LASTOVICA, A. J., ATHERTON, J. C. & LOUW, J. A. (1999) Heterogeneity in the Helicobacter pylori vacA and cagA genes: association with gastroduodenal disease in South Africa? *Gut,* 45**,** 499-502.

KIDO, M., YAMANAKA, K., MITANI, T., NIKI, H., OGURA, T. & HIRAGA, S. (1996) RNase E polypeptides lacking a carboxyl-terminal half suppress a mukB mutation in Escherichia coli. *J Bacteriol,* 178**,** 3917-25.

KIM, H., LIM, J. W. & KIM, K. H. (2001) Helicobacter pylori-induced expression of interleukin-8 and cyclooxygenase-2 in AGS gastric epithelial cells: mediation by nuclear factor-kappaB. *Scand J Gastroenterol,* 36**,** 706-16.

KIM, J. M., KIM, J. S., LEE, J. Y., KIM, Y. J., YOUN, H. J., KIM, I. Y., CHEE, Y. J., OH, Y. K., KIM, N., JUNG, H. C. & SONG, I. S. (2007) Vacuolating cytotoxin in Helicobacter pylori water-soluble proteins upregulates chemokine expression in human eosinophils via Ca2+ influx, mitochondrial reactive oxygen intermediates, and NF-kappaB activation. *Infection & Immunity,* 75**,** 3373-81.

KIM, J. S., CHANG, J. H., CHUNG, S. I. & YUM, J. S. (1999) Molecular cloning and characterization of the Helicobacter pylori fliD gene, an essential factor in flagellar structure and motility. *J Bacteriol,* 181**,** 6969-76.

KJELLEBERG, S., ALBERTSON, N., FLARDH, K., HOLMQUIST, L., JOUPER-JAAN, A., MAROUGA, R., OSTLING, J., SVENBLAD, B. & WEICHART, D. (1993) How do non-differentiating bacteria adapt to starvation? *Antonie Van Leeuwenhoek,* 63**,** 333-41.

KUIPERS, E. J., LEE, A., KLINKENBERG-KNOL, E. C. & MEUWISSEN, S. G. (1995a) Review article: the development of atrophic gastritis--Helicobacter pylori and the effects of acid suppressive therapy. *Aliment Pharmacol Ther,* 9**,** 331-40.

KUIPERS, E. J., LUNDELL, L., KLINKENBERG-KNOL, E. C., HAVU, N., FESTEN, H. P., LIEDMAN, B., LAMERS, C. B., JANSEN, J. B., DALENBACK, J., SNEL, P., NELIS, G. F. & MEUWISSEN, S. G. (1996) Atrophic gastritis and Helicobacter pylori infection in patients with reflux esophagitis treated with omeprazole or fundoplication. *N Engl J Med,* 334**,** 1018-22.

KUIPERS, E. J. & MEIJER, G. A. (2000) Helicobacter pylori gastritis in Africa. *Eur J Gastroenterol Hepatol,* 12**,** 601-3.

KUIPERS, E. J., PENA, A. S. & MEUWISSEN, S. G. (1995b) [Helicobacter pylori infection as causal factor in the development of carcinoma and lymphoma of the stomach; report WHO consensus conference]. *Ned Tijdschr Geneeskd,* 139**,** 709-12.

KUIPERS, E. J., PEREZ-PEREZ, G. I., MEUWISSEN, S. G. & BLASER, M. J. (1995c) Helicobacter pylori and atrophic gastritis: importance of the cagA status. *J Natl Cancer Inst,* 87**,** 1777-80.

KUIPERS, E. J., SCHENK, B. E., KLINKENBERG-KNOL, E. C. & MEUWISSEN, S. G. (1999) Helicobacter pylori and omeprazole therapy in GERD. *Am J Gastroenterol,* 94**,** 3378-9.

KUIPERS, E. J., SCHENK, B. E. & MEUWISSEN, S. G. (1995d) Helicobacter pylori: who is positive and who is not? *Eur J Gastroenterol Hepatol,* 7**,** 533-6.

KUIPERS, E. J., THIJS, J. C. & FESTEN, H. P. (1995e) The prevalence of Helicobacter pylori in peptic ulcer disease. *Aliment Pharmacol Ther,* 9 Suppl 2**,** 59-69.

KUIPERS, E. J., UYTERLINDE, A. M., PENA, A. S., HAZENBERG, H. J., BLOEMENA, E., LINDEMAN, J., KLINKENBERG-KNOL, E. C. & MEUWISSEN, S. G. (1995f) Increase of Helicobacter pylori-associated

corpus gastritis during acid suppressive therapy: implications for long-term safety. *Am J Gastroenterol,* 90**,** 1401-6.

KUNST, F., OGASAWARA, N., MOSZER, I., ALBERTINI, A. M., ALLONI, G., AZEVEDO, V., BERTERO, M. G., BESSIERES, P., BOLOTIN, A., BORCHERT, S., BORRISS, R., BOURSIER, L., BRANS, A., BRAUN, M., BRIGNELL, S. C., BRON, S., BROUILLET, S., BRUSCHI, C. V., CALDWELL, B., CAPUANO, V., CARTER, N. M., CHOI, S. K., CODANI, J. J., CONNERTON, I. F., DANCHIN, A. & ET AL. (1997) The complete genome sequence of the gram-positive bacterium Bacillus subtilis. *Nature,* 390**,** 249-56.

KURODA, M. I. & YANOFSKY, C. (1984) Evidence for the transcript secondary structures predicted to regulate transcription attenuation in the trp operon. *J Biol Chem,* 259**,** 12838-43.

KUSHNER, S. R. (2002) mRNA decay in Escherichia coli comes of age. *J Bacteriol,* 184**,** 4658-65; discussion 4657.

KUSTERS, J. G., GERRITS, M. M., VAN STRIJP, J. A. & VANDENBROUCKE-GRAULS, C. M. (1997) Coccoid forms of Helicobacter pylori are the morphologic manifestation of cell death. *Infect Immun,* 65**,** 3672-9.

LADOUKAKIS, E. D. & EYRE-WALKER, A. (2008) The excess of small inverted repeats in prokaryotes. *J Mol Evol,* 67**,** 291-300.

LANZAVECCHIA, S., BELLON, P. L., LUPETTI, P., DALLAI, R., RAPPUOLI, R. & TELFORD, J. L. (1998) Three-dimensional reconstruction of metal replicas of the Helicobacter pylori vacuolating cytotoxin. *Journal of Structural Biology,* 121**,** 9-18.

LAUREN, P. (1965) The Two Histological Main Types of Gastric Carcinoma: Diffuse and So-Called Intestinal-Type Carcinoma. An Attempt at a Histo-Clinical Classification. *Acta Pathol Microbiol Scand,* 64**,** 31-49.

LEHMANN, F. S., TERRACCIANO, L., CARENA, I., BAERISWYL, C., DREWE, J., TORNILLO, L., DE LIBERO, G. & BEGLINGER, C. (2002) In situ correlation of cytokine secretion and apoptosis in Helicobacter pylori-associated gastritis. *Am J Physiol Gastrointest Liver Physiol,* 283**,** G481-8.

LETLEY, D. P. & ATHERTON, J. C. (2000) Natural diversity in the N terminus of the mature vacuolating cytotoxin of Helicobacter pylori determines cytotoxin activity. *Journal of Bacteriology,* 182**,** 3278-80.

LETLEY, D. P., LASTOVICA, A., LOUW, J. A., HAWKEY, C. J. & ATHERTON, J. C. (1999) Allelic diversity of the Helicobacter pylori vacuolating cytotoxin gene in South Africa: rarity of the vacA s1a genotype and natural occurrence of an s2/m1 allele. *Journal of Clinical Microbiology,* 37**,** 1203-5.

LETLEY, D. P., RHEAD, J. L., TWELLS, R. J., DOVE, B. & ATHERTON, J. C. (2003) Determinants of non-toxicity in the gastric pathogen Helicobacter pylori. *Journal of Biological Chemistry,* 278**,** 26734-41.

LEUNK, R. D., JOHNSON, P. T., DAVID, B. C., KRAFT, W. G. & MORGAN, D. R. (1988) Cytotoxic activity in broth-culture filtrates of Campylobacter pylori. *J Med Microbiol,* 26**,** 93-9.

LEYING, H., SUERBAUM, S., GEIS, G. & HAAS, R. (1992) Cloning and genetic characterization of a Helicobacter pylori flagellin gene. *Mol Microbiol,* 6**,** 2863-74.

LI, H. & NICHOLSON, A. W. (1996) Defining the enzyme binding domain of a ribonuclease III processing signal. Ethylation interference and hydroxyl radical footprinting using catalytically inactive RNase III mutants. *EMBO J,* 15**,** 1421-33.

LOVETT, S. T. (2004) Encoded errors: mutations and rearrangements mediated by misalignment at repetitive DNA sequences. *Mol Microbiol,* 52**,** 1243-53.

LU, H., YAMAOKA, Y. & GRAHAM, D. Y. (2005) Helicobacter pylori virulence factors: facts and fantasies.[see comment]. *Current Opinion in Gastroenterology,* 21**,** 653-9.

LUCAS, B., BUMANN, D., WALDUCK, A., KOESLING, J., DEVELIOGLU, L., MEYER, T. F. & AEBISCHER, T. (2001) Adoptive transfer of CD4+ T cells specific for subunit A of Helicobacter pylori urease reduces H. pylori stomach colonization in mice in the absence of interleukin-4 (IL-4)/IL-13 receptor signaling. *Infect Immun,* 69**,** 1714-21.

LUNDSTROM, A. M. & BOLIN, I. (2000) A 26 kDa protein of helicobacter pylori shows alkyl hydroperoxide reductase (AhpC) activity and the mono-cistronic transcription of the gene is affected by pH. *Microb Pathog,* 29**,** 257-66.

LUPETTI, P., HEUSER, J. E., MANETTI, R., MASSARI, P., LANZAVECCHIA, S., BELLON, P. L., DALLAI, R., RAPPUOLI, R. & TELFORD, J. L. (1996) Oligomeric and subunit structure of the Helicobacter pylori vacuolating cytotoxin. *Journal of Cell Biology,* 133**,** 801-7.

MACKIE, G. A. (1991) Specific endonucleolytic cleavage of the mRNA for ribosomal protein S20 of Escherichia coli requires the product of the ams gene in vivo and in vitro. *J Bacteriol,* 173**,** 2488-97.

MACKIE, G. A. & GENEREAUX, J. L. (1993) The role of RNA structure in determining RNase E-dependent cleavage sites in the mRNA for ribosomal protein S20 in vitro. *J Mol Biol,* 234**,** 998-1012.

MAEDA, S., OGURA, K., YOSHIDA, H., KANAI, F., IKENOUE, T., KATO, N., SHIRATORI, Y. & OMATA, M. (1998) Major virulence factors, VacA and CagA, are commonly positive in Helicobacter pylori isolates in Japan. *Gut,* 42**,** 338-43.

MAEDA, S., YOSHIDA, H., IKENOUE, T., OGURA, K., KANAI, F., KATO, N., SHIRATORI, Y. & OMATA, M. (1999) Structure of cag pathogenicity island in Japanese Helicobacter pylori isolates. *Gut,* 44**,** 336-41.

MAHDAVI, J., BOREN, T., VANDENBROUCKE-GRAULS, C. & APPELMELK, B. J. (2003) Limited role of lipopolysaccharide Lewis antigens in adherence of Helicobacter pylori to the human gastric epithelium. *Infect Immun,* 71**,** 2876-80.

MAHDAVI, J., SONDEN, B., HURTIG, M., OLFAT, F. O., FORSBERG, L., ROCHE, N., ANGSTROM, J., LARSSON, T., TENEBERG, S., KARLSSON, K. A., ALTRAJA, S., WADSTROM, T., KERSULYTE,

D., BERG, D. E., DUBOIS, A., PETERSSON, C., MAGNUSSON, K. E., NORBERG, T., LINDH, F., LUNDSKOG, B. B., ARNQVIST, A., HAMMARSTROM, L. & BOREN, T. (2002) Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. *Science,* 297**,** 573-8.

MANNICK, E. E., BRAVO, L. E., ZARAMA, G., REALPE, J. L., ZHANG, X. J., RUIZ, B., FONTHAM, E. T., MERA, R., MILLER, M. J. & CORREA, P. (1996) Inducible nitric oxide synthase, nitrotyrosine, and apoptosis in Helicobacter pylori gastritis: effect of antibiotics and antioxidants. *Cancer Res,* 56**,** 3238-43.

MARCHETTI, M., ARICO, B., BURRONI, D., FIGURA, N., RAPPUOLI, R. & GHIARA, P. (1995) Development of a mouse model of Helicobacter pylori infection that mimics human disease. *Science,* 267**,** 1655-8.

MARSHALL, B. J. & WARREN, J. R. (1984) Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet,* 1**,** 1311-5.

MATSUNAGA, J., DYER, M., SIMONS, E. L. & SIMONS, R. W. (1996a) Expression and regulation of the rnc and pdxJ operons of Escherichia coli. *Mol Microbiol,* 22**,** 977-89.

MATSUNAGA, J., SIMONS, E. L. & SIMONS, R. W. (1996b) RNase III autoregulation: structure and function of rncO, the posttranscriptional "operator". *RNA,* 2**,** 1228-40.

MCCLAIN, M. S., CAO, P. & COVER, T. L. (2001) Amino-terminal hydrophobic region of Helicobacter pylori vacuolating cytotoxin (VacA) mediates transmembrane protein dimerization. *Infection & Immunity,* 69**,** 1181-4.

MCCLAIN, M. S., SCHRAW, W., RICCI, V., BOQUET, P. & COVER, T. L. (2000) Acid activation of Helicobacter pylori vacuolating cytotoxin (VacA) results in toxin internalization by eukaryotic cells. *Molecular Microbiology,* 37**,** 433-42.

MCDOWALL, K. J., LIN-CHAO, S. & COHEN, S. N. (1994) A+U content rather than a particular nucleotide order determines the specificity of RNase E cleavage. *J Biol Chem,* 269**,** 10790-6.

MELIN, L., RUTBERG, L. & VON GABAIN, A. (1989) Transcriptional and posttranscriptional control of the Bacillus subtilis succinate dehydrogenase operon. *J Bacteriol,* 171**,** 2110-5.

MENAKER, R. J., CEPONIS, P. J. & JONES, N. L. (2004) Helicobacter pylori induces apoptosis of macrophages in association with alterations in the mitochondrial pathway. *Infect Immun,* 72**,** 2889-98.

MERRELL, D. S., GOODRICH, M. L., OTTO, G., TOMPKINS, L. S. & FALKOW, S. (2003a) pH-regulated gene expression of the gastric pathogen Helicobacter pylori. *Infect Immun,* 71**,** 3529-39.

MERRELL, D. S., THOMPSON, L. J., KIM, C. C., MITCHELL, H., TOMPKINS, L. S., LEE, A. & FALKOW, S. (2003b) Growth phase-dependent response of Helicobacter pylori to iron starvation. *Infect Immun,* 71**,** 6510-25.

MOHAMMADI, M., NEDRUD, J., REDLINE, R., LYCKE, N. & CZINN, S. J. (1997) Murine CD4 T-cell response to Helicobacter infection: TH1

cells enhance gastritis and TH2 cells reduce bacterial load. *Gastroenterology,* 113**,** 1848-57.

MOLINARI, M., GALLI, C., DE BERNARD, M., NORAIS, N., RUYSSCHAERT, J. M., RAPPUOLI, R. & MONTECUCCO, C. (1998a) The acid activation of Helicobacter pylori toxin VacA: structural and membrane binding studies. *Biochem Biophys Res Commun,* 248**,** 334-40.

MOLINARI, M., GALLI, C., NORAIS, N., TELFORD, J. L., RAPPUOLI, R., LUZIO, J. P. & MONTECUCCO, C. (1997) Vacuoles induced by Helicobacter pylori toxin contain both late endosomal and lysosomal markers. *J Biol Chem,* 272**,** 25339-44.

MOLINARI, M., SALIO, M., GALLI, C., NORAIS, N., RAPPUOLI, R., LANZAVECCHIA, A. & MONTECUCCO, C. (1998b) Selective inhibition of Ii-dependent antigen presentation by Helicobacter pylori toxin VacA. *J Exp Med,* 187**,** 135-40.

MONSTEIN, H. J. & JONASSON, J. (2001) Differential virulence-gene mRNA expression in coccoid forms of Helicobacter pylori. *Biochem Biophys Res Commun,* 285**,** 530-6.

MONTEIRO, M. A., CHAN, K. H., RASKO, D. A., TAYLOR, D. E., ZHENG, P. Y., APPELMELK, B. J., WIRTH, H. P., YANG, M., BLASER, M. J., HYNES, S. O., MORAN, A. P. & PERRY, M. B. (1998) Simultaneous expression of type 1 and type 2 Lewis blood group antigens by Helicobacter pylori lipopolysaccharides. Molecular mimicry between h. pylori lipopolysaccharides and human gastric epithelial cell surface glycoforms. *J Biol Chem,* 273**,** 11533-43.

MOORE, K. E. & MIURA, S. (1987) A small hydrophobic domain anchors leader peptidase to the cytoplasmic membrane of Escherichia coli. *J Biol Chem,* 262**,** 8806-13.

MOSS, S. F. & BLASER, M. J. (2005) Mechanisms of disease: Inflammation and the origins of cancer. *Nat Clin Pract Oncol,* 2**,** 90-7; quiz 1 p following 113.

MOSS, S. F., CALAM, J., AGARWAL, B., WANG, S. & HOLT, P. R. (1996) Induction of gastric epithelial apoptosis by Helicobacter pylori. *Gut,* 38**,** 498-501.

NARAYANAN, G. L. (2005) Determinants of vacuolating cytotxin production in the gastric pathogen Helicobacter pylori. *Institute of Infection, Immunity and Inflammation.* Nottingham., University of Nottingham.

NAUMANN, M. & CRABTREE, J. E. (2004) Helicobacter pylori-induced epithelial cell signalling in gastric carcinogenesis. *Trends Microbiol,* 12**,** 29-36.

NEU, B., RANDLKOFER, P., NEUHOFER, M., VOLAND, P., MAYERHOFER, A., GERHARD, M., SCHEPP, W. & PRINZ, C. (2002) Helicobacter pylori induces apoptosis of rat gastric parietal cells. *Am J Physiol Gastrointest Liver Physiol,* 283**,** G309-18.

NIELSEN, H., ENGELBRECHT, J., BRUNAK, S. & VON HEIJNE, G. (1997a) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng,* 10**,** 1-6.

NIELSEN, H., ENGELBRECHT, J., BRUNAK, S. & VON HEIJNE, G. (1997b) A neural network method for identification of prokaryotic and

eukaryotic signal peptides and prediction of their cleavage sites. *Int J Neural Syst,* 8**,** 581-99.

NILSSON, I. & VON HEIJNE, G. (1992) A signal peptide with a proline next to the cleavage site inhibits leader peptidase when present in a sec-independent protein. *FEBS Lett,* 299**,** 243-6.

NURNBERGER, T., BRUNNER, F., KEMMERLING, B. & PIATER, L. (2004) Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol Rev,* 198**,** 249-66.

ODENBREIT, S., TILL, M. & HAAS, R. (1996) Optimized BlaM-transposon shuttle mutagenesis of Helicobacter pylori allows the identification of novel genetic loci involved in bacterial virulence. *Mol Microbiol,* 20**,** 361-73.

ODENBREIT, S., TILL, M., HOFREUTER, D., FALLER, G. & HAAS, R. (1999) Genetic and functional characterization of the alpAB gene locus essential for the adhesion of Helicobacter pylori to human gastric tissue. *Mol Microbiol,* 31**,** 1537-48.

OGURA, K., MAEDA, S., NAKAO, M., WATANABE, T., TADA, M., KYUTOKU, T., YOSHIDA, H., SHIRATORI, Y. & OMATA, M. (2000) Virulence factors of Helicobacter pylori responsible for gastric diseases in Mongolian gerbil. *Journal of Experimental Medicine,* 192**,** 1601-10.

OTTEMANN, K. M. & LOWENTHAL, A. C. (2002) Helicobacter pylori uses motility for initial colonization and to attain robust infection. *Infect Immun,* 70**,** 1984-90.

PAGLIACCIA, C., DE BERNARD, M., LUPETTI, P., JI, X., BURRONI, D., COVER, T. L., PAPINI, E., RAPPUOLI, R., TELFORD, J. L. & REYRAT, J. M. (1998) The m2 form of the Helicobacter pylori cytotoxin has cell type-specific vacuolating activity. *Proc Natl Acad Sci U S A,* 95**,** 10212-7.

PAPINI, E., BUGNOLI, M., DE BERNARD, M., FIGURA, N., RAPPUOLI, R. & MONTECUCCO, C. (1993a) Bafilomycin A1 inhibits Helicobacter pylori-induced vacuolization of HeLa cells. *Mol Microbiol,* 7**,** 323-7.

PAPINI, E., DE BERNARD, M., BUGNOLI, M., MILIA, E., RAPPUOLI, R. & MONTECUCCO, C. (1993b) Cell vacuolization induced by Helicobacter pylori: inhibition by bafilomycins A1, B1, C1 and D. *FEMS Microbiol Lett,* 113**,** 155-9.

PAPINI, E., DE BERNARD, M., MILIA, E., BUGNOLI, M., ZERIAL, M., RAPPUOLI, R. & MONTECUCCO, C. (1994) Cellular vacuoles induced by Helicobacter pylori originate from late endosomal compartments. *Proc Natl Acad Sci U S A,* 91**,** 9720-4.

PAPINI, E., SATIN, B., BUCCI, C., DE BERNARD, M., TELFORD, J. L., MANETTI, R., RAPPUOLI, R., ZERIAL, M. & MONTECUCCO, C. (1997) The small GTP binding protein rab7 is essential for cellular vacuolation induced by Helicobacter pylori cytotoxin. *EMBO J,* 16**,** 15-24.

PAPINI, E., SATIN, B., DE BERNARD, M., MOLINARI, M., ARICO, B., GALLI, C., TELFORD, J. R., RAPPUOLI, R. & MONTECUCCO, C.

(1998) Action site and cellular effects of cytotoxin VacA produced by Helicobacter pylori. *Folia Microbiol (Praha),* 43**,** 279-84.

PAPINI, E., ZORATTI, M. & COVER, T. L. (2001) In search of the Helicobacter pylori VacA mechanism of action. *Toxicon,* 39**,** 1757-67.

PARKHILL, J., WREN, B. W., MUNGALL, K., KETLEY, J. M., CHURCHER, C., BASHAM, D., CHILLINGWORTH, T., DAVIES, R. M., FELTWELL, T., HOLROYD, S., JAGELS, K., KARLYSHEV, A. V., MOULE, S., PALLEN, M. J., PENN, C. W., QUAIL, M. A., RAJANDREAM, M. A., RUTHERFORD, K. M., VAN VLIET, A. H., WHITEHEAD, S. & BARRELL, B. G. (2000) The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences. *Nature,* 403**,** 665-8.

PARSONNET, J. (1994) Gastric adenocarcinoma and Helicobacter pylori infection. *West J Med,* 161**,** 60.

PARSONNET, J., FRIEDMAN, G. D., ORENTREICH, N. & VOGELMAN, H. (1997) Risk for gastric cancer in people with CagA positive or CagA negative Helicobacter pylori infection. *Gut,* 40**,** 297-301.

PARSONNET, J., FRIEDMAN, G. D., VANDERSTEEN, D. P., CHANG, Y., VOGELMAN, J. H., ORENTREICH, N. & SIBLEY, R. K. (1991a) Helicobacter pylori infection and the risk of gastric carcinoma. *N Engl J Med,* 325**,** 1127-31.

PARSONNET, J., HANSEN, S., RODRIGUEZ, L., GELB, A. B., WARNKE, R. A., JELLUM, E., ORENTREICH, N., VOGELMAN, J. H. & FRIEDMAN, G. D. (1994) Helicobacter pylori infection and gastric lymphoma. *N Engl J Med,* 330**,** 1267-71.

PARSONNET, J. & ISAACSON, P. G. (2004) Bacterial infection and MALT lymphoma. *N Engl J Med,* 350**,** 213-5.

PARSONNET, J., VANDERSTEEN, D., GOATES, J., SIBLEY, R. K., PRITIKIN, J. & CHANG, Y. (1991b) Helicobacter pylori infection in intestinal- and diffuse-type gastric adenocarcinomas. *J Natl Cancer Inst,* 83**,** 640-3.

PEEK, R. M., JR. & BLASER, M. J. (2002) Helicobacter pylori and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer,* 2**,** 28-37.

PEEK, R. M., JR., MILLER, G. G., THAM, K. T., PEREZ-PEREZ, G. I., ZHAO, X., ATHERTON, J. C. & BLASER, M. J. (1995) Heightened inflammatory response and cytokine expression in vivo to cagA+ Helicobacter pylori strains. *Lab Invest,* 73**,** 760-70.

PEEK, R. M., JR., THOMPSON, S. A., DONAHUE, J. P., THAM, K. T., ATHERTON, J. C., BLASER, M. J. & MILLER, G. G. (1998) Adherence to gastric epithelial cells induces expression of a Helicobacter pylori gene, iceA, that is associated with clinical outcome. *Proceedings of the Association of American Physicians,* 110**,** 531-44.

PERLMAN, D. & HALVORSON, H. O. (1983) A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *J Mol Biol,* 167**,** 391-409.

PESCI, E. C., COTTLE, D. L. & PICKETT, C. L. (1994) Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of Campylobacter jejuni. *Infect Immun,* 62**,** 2687-94.

PETERSSON, C., FORSBERG, M., ASPHOLM, M., OLFAT, F. O., FORSLUND, T., BOREN, T. & MAGNUSSON, K. E. (2006) Helicobacter pylori SabA adhesin evokes a strong inflammatory response in human neutrophils which is down-regulated by the neutrophil-activating protein. *Med Microbiol Immunol,* 195**,** 195-206.

PFAFFL, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res,* 29**,** e45.

PFLOCK, M., KENNARD, S., DELANY, I., SCARLATO, V. & BEIER, D. (2005) Acid-induced activation of the urease promoters is mediated directly by the ArsRS two-component system of Helicobacter pylori. *Infect Immun,* 73**,** 6437-45.

PFLOCK, M., KENNARD, S., FINSTERER, N. & BEIER, D. (2006) Acid-responsive gene regulation in the human pathogen Helicobacter pylori. *J Biotechnol,* 126**,** 52-60.

PHADNIS, S. H., ILVER, D., JANZON, L., NORMARK, S. & WESTBLOM, T. U. (1994) Pathological significance and molecular characterization of the vacuolating toxin gene of Helicobacter pylori. *Infect Immun,* 62**,** 1557-65.

PORWOLLIK, S., NOONAN, B. & O'TOOLE, P. W. (1999) Molecular characterization of a flagellar export locus of Helicobacter pylori. *Infect Immun,* 67**,** 2060-70.

QUEIROZ, D. M., MENDES, E. N., ROCHA, G. A., OLIVEIRA, A. M., OLIVEIRA, C. A., MAGALHAES, P. P., MOURA, S. B., CABRAL, M. M. & NOGUEIRA, A. M. (1998) cagA-positive Helicobacter pylori and risk for developing gastric carcinoma in Brazil. *Int J Cancer,* 78**,** 135-9.

QUISEL, J. D., LIN, D. C. & GROSSMAN, A. D. (1999) Control of development by altered localization of a transcription factor in B. subtilis. *Mol Cell,* 4**,** 665-72.

RAVNUM, S. & ANDERSSON, D. I. (2001) An adenosyl-cobalamin (coenzyme-B12)-repressed translational enhancer in the cob mRNA of Salmonella typhimurium. *Mol Microbiol,* 39**,** 1585-94.

REGNIER, P. & ARRAIANO, C. M. (2000) Degradation of mRNA in bacteria: emergence of ubiquitous features. *Bioessays,* 22**,** 235-44.

RESNEKOV, O., RUTBERG, L. & VON GABAIN, A. (1990) Changes in the stability of specific mRNA species in response to growth stage in Bacillus subtilis. *Proc Natl Acad Sci U S A,* 87**,** 8355-9.

REYRAT, J. M., LANZAVECCHIA, S., LUPETTI, P., DE BERNARD, M., PAGLIACCIA, C., PELICIC, V., CHARREL, M., ULIVIERI, C., NORAIS, N., JI, X., CABIAUX, V., PAPINI, E., RAPPUOLI, R. & TELFORD, J. L. (1999) 3D imaging of the 58 kDa cell binding subunit of the Helicobacter pylori cytotoxin. *J Mol Biol,* 290**,** 459-70.

RHEAD, J. L., LETLEY, D. P., MOHAMMADI, M., HUSSEIN, N., MOHAGHEGHI, M. A., ESHAGH HOSSEINI, M. & ATHERTON, J. C. (2007) A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer.[see comment]. *Gastroenterology,* 133**,** 926-36.

RICCI, V., SOMMI, P., FIOCCA, R., ROMANO, M., SOLCIA, E. & VENTURA, U. (1997) Helicobacter pylori vacuolating toxin

accumulates within the endosomal-vacuolar compartment of cultured gastric cells and potentiates the vacuolating activity of ammonia. *J Pathol,* 183**,** 453-9.

RITTIG, M. G., SHAW, B., LETLEY, D. P., THOMAS, R. J., ARGENT, R. H. & ATHERTON, J. C. (2003) Helicobacter pylori-induced homotypic phagosome fusion in human monocytes is independent of the bacterial vacA and cag status. *Cellular Microbiology,* 5**,** 887-99.

ROCCO, A. & NARDONE, G. (2007) Diet, H pylori infection and gastric cancer: evidence and controversies. *World J Gastroenterol,* 13**,** 2901-12.

ROMEO, T. (1998) Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. *Mol Microbiol,* 29**,** 1321-30.

ROMEO, T. & GONG, M. (1993) Genetic and physical mapping of the regulatory gene csrA on the Escherichia coli K-12 chromosome. *J Bacteriol,* 175**,** 5740-1.

RUDI, J., KOLB, C., MAIWALD, M., KUCK, D., SIEG, A., GALLE, P. R. & STREMMEL, W. (1998) Diversity of Helicobacter pylori vacA and cagA genes and relationship to VacA and CagA protein expression, cytotoxin production, and associated diseases.[see comment]. *Journal of Clinical Microbiology,* 36**,** 944-8.

RUDI, J., KOLB, C., MAIWALD, M., ZUNA, I., VON HERBAY, A., GALLE, P. R. & STREMMEL, W. (1997) Serum antibodies against Helicobacter pylori proteins VacA and CagA are associated with increased risk for gastric adenocarcinoma. *Digestive Diseases & Sciences,* 42**,** 1652-9.

SALAMA, N., GUILLEMIN, K., MCDANIEL, T. K., SHERLOCK, G., TOMPKINS, L. & FALKOW, S. (2000) A whole-genome microarray reveals genetic diversity among Helicobacter pylori strains. *Proc Natl Acad Sci U S A,* 97**,** 14668-73.

SALAMA, N. R., OTTO, G., TOMPKINS, L. & FALKOW, S. (2001) Vacuolating cytotoxin of Helicobacter pylori plays a role during colonization in a mouse model of infection. *Infection & Immunity,* 69**,** 730-6.

SALAUN, L., LINZ, B., SUERBAUM, S. & SAUNDERS, N. J. (2004) The diversity within an expanded and redefined repertoire of phase-variable genes in Helicobacter pylori. *Microbiology,* 150**,** 817-30.

SAN MILLAN, J. L., BOYD, D., DALBEY, R., WICKNER, W. & BECKWITH, J. (1989) Use of phoA fusions to study the topology of the Escherichia coli inner membrane protein leader peptidase. *J Bacteriol,* 171**,** 5536-41.

SANDLER, P. & WEISBLUM, B. (1988) Erythromycin-induced stabilization of ermA messenger RNA in Staphylococcus aureus and Bacillus subtilis. *J Mol Biol,* 203**,** 905-15.

SANDLER, P. & WEISBLUM, B. (1989) Erythromycin-induced ribosome stall in the ermA leader: a barricade to 5'-to-3' nucleolytic cleavage of the ermA transcript. *J Bacteriol,* 171**,** 6680-8.

SCHMITT, W. & HAAS, R. (1994) Genetic analysis of the Helicobacter pylori vacuolating cytotoxin: structural similarities with the IgA protease type of exported protein. *Molecular Microbiology,* 12**,** 307-19.

SCHMITZ, A., JOSENHANS, C. & SUERBAUM, S. (1997) Cloning and characterization of the Helicobacter pylori flbA gene, which codes for a membrane protein involved in coordinated expression of flagellar genes. *J Bacteriol,* 179**,** 987-97.

SCHREIBER, S., KONRADT, M., GROLL, C., SCHEID, P., HANAUER, G., WERLING, H. O., JOSENHANS, C. & SUERBAUM, S. (2004) The spatial orientation of Helicobacter pylori in the gastric mucus. *Proc Natl Acad Sci U S A,* 101**,** 5024-9.

SCHWABE, H., STEIN, U. & WALTHER, W. (2000) High-copy cDNA amplification of minimal total RNA quantities for gene expression analyses. *Mol Biotechnol,* 14**,** 165-72.

SCOTT, D. R., WEEKS, D., HONG, C., POSTIUS, S., MELCHERS, K. & SACHS, G. (1998) The role of internal urease in acid resistance of Helicobacter pylori. *Gastroenterology,* 114**,** 58-70.

SELBACH, M., MOESE, S., HAUCK, C. R., MEYER, T. F. & BACKERT, S. (2002) Src is the kinase of the Helicobacter pylori CagA protein in vitro and in vivo. *J Biol Chem,* 277**,** 6775-8.

SHEN, L. M., LEE, J. I., CHENG, S. Y., JUTTE, H., KUHN, A. & DALBEY, R. E. (1991) Use of site-directed mutagenesis to define the limits of sequence variation tolerated for processing of the M13 procoat protein by the Escherichia coli leader peptidase. *Biochemistry,* 30**,** 11775-81.

SHIMOYAMA, T., EVERETT, S. M., FUKUDA, S., AXON, A. T., DIXON, M. F. & CRABTREE, J. E. (2001) Influence of smoking and alcohol on gastric chemokine mRNA expression in patients with Helicobacter pylori infection. *J Clin Pathol,* 54**,** 332-4.

SIPPONEN, P. & CORREA, P. (2002) Delayed rise in incidence of gastric cancer in females results in unique sex ratio (M/F) pattern: etiologic hypothesis. *Gastric Cancer,* 5**,** 213-9.

SIPPONEN, P., KEKKI, M., HAAPAKOSKI, J., IHAMAKI, T. & SIURALA, M. (1985) Gastric cancer risk in chronic atrophic gastritis: statistical calculations of cross-sectional data. *Int J Cancer,* 35**,** 173-7.

SMITH, M. F., JR., MITCHELL, A., LI, G., DING, S., FITZMAURICE, A. M., RYAN, K., CROWE, S. & GOLDBERG, J. B. (2003) Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for Helicobacter pylori-induced NF-kappa B activation and chemokine expression by epithelial cells. *J Biol Chem,* 278**,** 32552-60.

SMOOT, D. T., MOBLEY, H. L., CHIPPENDALE, G. R., LEWISON, J. F. & RESAU, J. H. (1990) Helicobacter pylori urease activity is toxic to human gastric epithelial cells. *Infect Immun,* 58**,** 1992-4.

SMYTHIES, L. E., WAITES, K. B., LINDSEY, J. R., HARRIS, P. R., GHIARA, P. & SMITH, P. D. (2000) Helicobacter pylori-induced mucosal inflammation is Th1 mediated and exacerbated in IL-4, but not IFN-gamma, gene-deficient mice. *J Immunol,* 165**,** 1022-9.

SOLNICK, J. V., HANSEN, L. M. & SYVANEN, M. (1997) The major sigma factor (RpoD) from Helicobacter pylori and other gram-negative bacteria shows an enhanced rate of divergence. *J Bacteriol,* 179**,** 6196-200.

SPOHN, G., DELANY, I., RAPPUOLI, R. & SCARLATO, V. (2002) Characterization of the HspR-mediated stress response in Helicobacter pylori. *J Bacteriol,* 184**,** 2925-30.

SPOHN, G. & SCARLATO, V. (1999a) The autoregulatory HspR repressor protein governs chaperone gene transcription in Helicobacter pylori. *Mol Microbiol,* 34**,** 663-74.

SPOHN, G. & SCARLATO, V. (1999b) Motility of Helicobacter pylori is coordinately regulated by the transcriptional activator FlgR, an NtrC homolog. *J Bacteriol,* 181**,** 593-9.

STEIN, M., BAGNOLI, F., HALENBECK, R., RAPPUOLI, R., FANTL, W. J. & COVACCI, A. (2002) c-Src/Lyn kinases activate Helicobacter pylori CagA through tyrosine phosphorylation of the EPIYA motifs. *Mol Microbiol,* 43**,** 971-80.

STEUERWALD, N., COHEN, J., HERRERA, R. J. & BRENNER, C. A. (1999) Analysis of gene expression in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR. *Mol Hum Reprod,* 5**,** 1034-9.

SUERBAUM, S., JOSENHANS, C. & LABIGNE, A. (1993) Cloning and genetic characterization of the Helicobacter pylori and Helicobacter mustelae flaB flagellin genes and construction of H. pylori flaA- and flaB-negative mutants by electroporation-mediated allelic exchange. *J Bacteriol,* 175**,** 3278-88.

SUPAJATURA, V., USHIO, H., WADA, A., YAHIRO, K., OKUMURA, K., OGAWA, H., HIRAYAMA, T. & RA, C. (2002) Cutting edge: VacA, a vacuolating cytotoxin of Helicobacter pylori, directly activates mast cells for migration and production of proinflammatory cytokines. *Journal of Immunology,* 168**,** 2603-7.

SZABO, I., BRUTSCHE, S., TOMBOLA, F., MOSCHIONI, M., SATIN, B., TELFORD, J. L., RAPPUOLI, R., MONTECUCCO, C., PAPINI, E. & ZORATTI, M. (1999) Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of Helicobacter pylori is required for its biological activity. *EMBO J,* 18**,** 5517-27.

SZCZEBARA, F., DHAENENS, L., ARMAND, S. & HUSSON, M. O. (1999) Regulation of the transcription of genes encoding different virulence factors in Helicobacter pylori by free iron. *FEMS Microbiol Lett,* 175**,** 165-70.

TAKATA, T., EL-OMAR, E., CAMORLINGA, M., THOMPSON, S. A., MINOHARA, Y., ERNST, P. B. & BLASER, M. J. (2002) Helicobacter pylori does not require Lewis X or Lewis Y expression to colonize C3H/HeJ mice. *Infect Immun,* 70**,** 3073-9.

TELFORD, J. L., COVACCI, A., GHIARA, P., MONTECUCCO, C. & RAPPUOLI, R. (1994a) Unravelling the pathogenic role of Helicobacter pylori in peptic ulcer: potential new therapies and vaccines. *Trends Biotechnol,* 12**,** 420-6.

TELFORD, J. L., GHIARA, P., DELL'ORCO, M., COMANDUCCI, M., BURRONI, D., BUGNOLI, M., TECCE, M. F., CENSINI, S., COVACCI, A., XIANG, Z. & ET AL. (1994b) Gene structure of the Helicobacter pylori cytotoxin and evidence of its key role in gastric disease. *J Exp Med,* 179**,** 1653-58.

THOMPSON, L. J., MERRELL, D. S., NEILAN, B. A., MITCHELL, H., LEE, A. & FALKOW, S. (2003) Gene expression profiling of Helicobacter pylori reveals a growth-phase-dependent switch in virulence gene expression. *Infect Immun,* 71**,** 2643-55.

TOMB, J. F., WHITE, O., KERLAVAGE, A. R., CLAYTON, R. A., SUTTON, G. G., FLEISCHMANN, R. D., KETCHUM, K. A., KLENK, H. P., GILL, S., DOUGHERTY, B. A., NELSON, K., QUACKENBUSH, J., ZHOU, L., KIRKNESS, E. F., PETERSON, S., LOFTUS, B., RICHARDSON, D., DODSON, R., KHALAK, H. G., GLODEK, A., MCKENNEY, K., FITZEGERALD, L. M., LEE, N., ADAMS, M. D., HICKEY, E. K., BERG, D. E., GOCAYNE, J. D., UTTERBACK, T. R., PETERSON, J. D., KELLEY, J. M., COTTON, M. D., WEIDMAN, J. M., FUJII, C., BOWMAN, C., WATTHEY, L., WALLIN, E., HAYES, W. S., BORODOVSKY, M., KARP, P. D., SMITH, H. O., FRASER, C. M. & VENTER, J. C. (1997) The complete genome sequence of the gastric pathogen Helicobacter pylori. *Nature,* 388**,** 539-47.

TOMBOLA, F., CARLESSO, C., SZABO, I., DE BERNARD, M., REYRAT, J. M., TELFORD, J. L., RAPPUOLI, R., MONTECUCCO, C., PAPINI, E. & ZORATTI, M. (1999) Helicobacter pylori vacuolating toxin forms anion-selective channels in planar lipid bilayers: possible implications for the mechanism of cellular vacuolation. *Biophys J,* 76**,** 1401-9.

TOMBOLA, F., MORBIATO, L., DEL GIUDICE, G., RAPPUOLI, R., ZORATTI, M. & PAPINI, E. (2001) The Helicobacter pylori VacA toxin is a urea permease that promotes urea diffusion across epithelia. *J Clin Invest,* 108**,** 929-37.

TOROK, A. M., BOUTON, A. H. & GOLDBERG, J. B. (2005) Helicobacter pylori induces interleukin-8 secretion by Toll-like receptor 2- and Toll-like receptor 5-dependent and -independent pathways. *Infect Immun,* 73**,** 1523-31.

TSCHANTZ, W. R., PAETZEL, M., CAO, G., SUCIU, D., INOUYE, M. & DALBEY, R. E. (1995) Characterization of a soluble, catalytically active form of Escherichia coli leader peptidase: requirement of detergent or phospholipid for optimal activity. *Biochemistry,* 34**,** 3935-41.

TSUDA, M., KARITA, M., MIZOTE, T., MORSHED, M. G., OKITA, K. & NAKAZAWA, T. (1994) Essential role of Helicobacter pylori urease in gastric colonization: definite proof using a urease-negative mutant constructed by gene replacement. *Eur J Gastroenterol Hepatol,* 6 Suppl 1**,** S49-52.

TSUTSUMI, R., HIGASHI, H., HIGUCHI, M., OKADA, M. & HATAKEYAMA, M. (2003) Attenuation of Helicobacter pylori CagA x SHP-2 signaling by interaction between CagA and C-terminal Src kinase. *J Biol Chem,* 278**,** 3664-70.

UMIT, H., TEZEL, A., BUKAVAZ, S., UNSAL, G., OTKUN, M., SOYLU, A. R., TUCER, D. & BILGI, S. (2009) The relationship between virulence factors of Helicobacter pylori and severity of gastritis in infected patients. *Digestive Diseases & Sciences,* 54**,** 103-10.

UNNIRAMAN, S., CHATTERJI, M. & NAGARAJA, V. (2002a) A hairpin near the 5' end stabilises the DNA gyrase mRNA in Mycobacterium smegmatis. *Nucleic Acids Res,* 30**,** 5376-81.

UNNIRAMAN, S., PRAKASH, R. & NAGARAJA, V. (2002b) Conserved economics of transcription termination in eubacteria. *Nucleic Acids Res,* 30**,** 675-84.

VAN AMSTERDAM, K., VAN VLIET, A. H., KUSTERS, J. G., FELLER, M., DANKERT, J. & VAN DER ENDE, A. (2003) Induced Helicobacter pylori vacuolating cytotoxin VacA expression after initial colonisation of human gastric epithelial cells. *FEMS Immunology & Medical Microbiology,* 39**,** 251-6.

VAN BELKUM, A. (1999) The role of short sequence repeats in epidemiologic typing. *Curr Opin Microbiol,* 2**,** 306-11.

VAN DOORN, L. J., FIGUEIREDO, C., SANNA, R., PENA, S., MIDOLO, P., NG, E. K., ATHERTON, J. C., BLASER, M. J. & QUINT, W. G. (1998) Expanding allelic diversity of Helicobacter pylori vacA.[erratum appears in J Clin Microbiol 2000 Jun;38(6):2464]. *Journal of Clinical Microbiology,* 36**,** 2597-603.

VAN DOORN, N. E., NAMAVAR, F., SPARRIUS, M., STOOF, J., VAN REES, E. P., VAN DOORN, L. J. & VANDENBROUCKE-GRAULS, C. M. (1999) Helicobacter pylori-associated gastritis in mice is host and strain specific. *Infection & Immunity,* 67**,** 3040-6.

VAN VLIET, A. H., ERNST, F. D. & KUSTERS, J. G. (2004a) NikR-mediated regulation of Helicobacter pylori acid adaptation. *Trends Microbiol,* 12**,** 489-94.

VAN VLIET, A. H., KUIPERS, E. J., STOOF, J., POPPELAARS, S. W. & KUSTERS, J. G. (2004b) Acid-responsive gene induction of ammonia-producing enzymes in Helicobacter pylori is mediated via a metal-responsive repressor cascade. *Infect Immun,* 72**,** 766-73.

VAN VLIET, A. H., POPPELAARS, S. W., DAVIES, B. J., STOOF, J., BERESWILL, S., KIST, M., PENN, C. W., KUIPERS, E. J. & KUSTERS, J. G. (2002) NikR mediates nickel-responsive transcriptional induction of urease expression in Helicobacter pylori. *Infect Immun,* 70**,** 2846-52.

VEIGA, E., DE LORENZO, V. & FERNANDEZ, L. A. (2004) Structural tolerance of bacterial autotransporters for folded passenger protein domains. *Mol Microbiol,* 52**,** 1069-80.

VIALA, J., CHAPUT, C., BONECA, I. G., CARDONA, A., GIRARDIN, S. E., MORAN, A. P., ATHMAN, R., MEMET, S., HUERRE, M. R., COYLE, A. J., DISTEFANO, P. S., SANSONETTI, P. J., LABIGNE, A., BERTIN, J., PHILPOTT, D. J. & FERRERO, R. L. (2004) Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat Immunol,* 5**,** 1166-74.

VINION-DUBIEL, A. D., MCCLAIN, M. S., CZAJKOWSKY, D. M., IWAMOTO, H., YE, D., CAO, P., SCHRAW, W., SZABO, G., BLANKE, S. R., SHAO, Z. & COVER, T. L. (1999) A dominant negative mutant of Helicobacter pylori vacuolating toxin (VacA) inhibits VacA-induced cell vacuolation. *J Biol Chem,* 274**,** 37736-42.

VON HEIJNE, G. (1983) Patterns of amino acids near signal-sequence cleavage sites. *Eur J Biochem,* 133**,** 17-21.

VON HEIJNE, G. (1984) How signal sequences maintain cleavage specificity. *J Mol Biol,* 173**,** 243-51.

WAIDNER, B., MELCHERS, K., STAHLER, F. N., KIST, M. & BERESWILL, S. (2005) The Helicobacter pylori CrdRS two-component regulation system (HP1364/HP1365) is required for copper-mediated induction of the copper resistance determinant CrdA. *J Bacteriol,* 187**,** 4683-8.

WANG, Y. & TAYLOR, D. E. (1990) Natural transformation in Campylobacter species. *J Bacteriol,* 172**,** 949-55.

WASHIO, T., SASAYAMA, J. & TOMITA, M. (1998) Analysis of complete genomes suggests that many prokaryotes do not rely on hairpin formation in transcription termination. *Nucleic Acids Res,* 26**,** 5456-63.

WEN, Y., FENG, J., SCOTT, D. R., MARCUS, E. A. & SACHS, G. (2006) Involvement of the HP0165-HP0166 two-component system in expression of some acidic-pH-upregulated genes of Helicobacter pylori. *J Bacteriol,* 188**,** 1750-61.

WEN, Y., MARCUS, E. A., MATRUBUTHAM, U., GLEESON, M. A., SCOTT, D. R. & SACHS, G. (2003) Acid-adaptive genes of Helicobacter pylori. *Infect Immun,* 71**,** 5921-39.

WILLHITE, D. C. & BLANKE, S. R. (2004) Helicobacter pylori vacuolating cytotoxin enters cells, localizes to the mitochondria, and induces mitochondrial membrane permeability changes correlated to toxin channel activity. *Cellular Microbiology,* 6**,** 143-54.

WOLFE, P. B., ZWIZINSKI, C. & WICKNER, W. (1983) Purification and characterization of leader peptidase from Escherichia coli. *Methods Enzymol,* 97**,** 40-6.

WOLFRAM, L., HAAS, E. & BAUERFEIND, P. (2006) Nickel represses the synthesis of the nickel permease NixA of Helicobacter pylori. *J Bacteriol,* 188**,** 1245-50.

WOO, E. Y., YEH, H., CHU, C. S., SCHLIENGER, K., CARROLL, R. G., RILEY, J. L., KAISER, L. R. & JUNE, C. H. (2002) Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol,* 168**,** 4272-6.

WORST, D. J., GERRITS, M. M., VANDENBROUCKE-GRAULS, C. M. & KUSTERS, J. G. (1998) Helicobacter pylori ribBA-mediated riboflavin production is involved in iron acquisition. *J Bacteriol,* 180**,** 1473-9.

XIA, B., KE, H., JIANG, W. & INOUYE, M. (2002) The Cold Box stem-loop proximal to the 5'-end of the Escherichia coli cspA gene stabilizes its mRNA at low temperature. *J Biol Chem,* 277**,** 6005-11.

YAHIRO, K., NIIDOME, T., KIMURA, M., HATAKEYAMA, T., AOYAGI, H., KURAZONO, H., IMAGAWA, K., WADA, A., MOSS, J. & HIRAYAMA, T. (1999) Activation of Helicobacter pylori VacA toxin by alkaline or acid conditions increases its binding to a 250-kDa receptor protein-tyrosine phosphatase beta. *J Biol Chem,* 274**,** 36693-9.

YAMAOKA, Y., KIKUCHI, S., EL-ZIMAITY, H. M., GUTIERREZ, O., OSATO, M. S. & GRAHAM, D. Y. (2002) Importance of Helicobacter

pylori oipA in clinical presentation, gastric inflammation, and mucosal interleukin 8 production. *Gastroenterology,* 123**,** 414-24.

YAMAOKA, Y., KODAMA, T., KITA, M., IMANISHI, J., KASHIMA, K. & GRAHAM, D. Y. (1998) Relationship of vacA genotypes of Helicobacter pylori to cagA status, cytotoxin production, and clinical outcome. *Helicobacter,* 3**,** 241-53.

YAMAOKA, Y., YAMAUCHI, K., OTA, H., SUGIYAMA, A., ISHIZONE, S., GRAHAM, D. Y., MARUTA, F., MURAKAMI, M. & KATSUYAMA, T. (2005) Natural history of gastric mucosal cytokine expression in Helicobacter pylori gastritis in Mongolian gerbils. *Infect Immun,* 73**,** 2205-12.

YE, D., WILLHITE, D. C. & BLANKE, S. R. (1999) Identification of the minimal intracellular vacuolating domain of the Helicobacter pylori vacuolating toxin. *J Biol Chem,* 274**,** 9277-82.

ZHANG, K. & NICHOLSON, A. W. (1997) Regulation of ribonuclease III processing by double-helical sequence antideterminants. *Proc Natl Acad Sci U S A,* 94**,** 13437-41.

ZHENG, P. Y. & JONES, N. L. (2003) Helicobacter pylori strains expressing the vacuolating cytotoxin interrupt phagosome maturation in macrophages by recruiting and retaining TACO (coronin 1) protein. *Cellular Microbiology,* 5**,** 25-40.

ZWIZINSKI, C., DATE, T. & WICKNER, W. (1981) Leader peptidase is found in both the inner and outer membranes of Escherichia coli. *J Biol Chem,* 256**,** 3593-7.