MICROBIAL PATHOGENICITY

***Helicobacter pylori* adherence to gastric epithelial cells: a role for non-adhesin virulence genes**

ZUN-WU ZHANG, NICK DORRELL*, BRENDAN W. WREN*, MICHAEL J. G. FARTHING†

Division of Surgery, Bristol Royal Infirmary, University of Bristol, Bristol, *Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London and †Faculty of Medicine, University of Glasgow, Glasgow, UK

Helicobacter pylori is a major aetiological agent in gastroduodenal disorders and adherence of the bacteria to the gastric mucosa is one of the initial stages of infection. Although a number of specific adhesins has been identified, other *H. pylori* virulence factors may play a role in adherence to gastric epithelial cells directly or through interaction with other adhesins. This study assessed the effect of 16 *H. pylori* virulence factors on the adherence of the bacteria to gastric AGS cells and on gastric epithelial cell cycle distribution. Defined isogenic *H. pylori* SS1 mutants were used. After co-incubation of gastric AGS cells and bacteria, adherence of *H. pylori* to AGS cells was visualised by immunofluorescence microscopy and quantified by flow cytometry. Cell cycle phase distribution was analysed by flow cytometry with propidium iodide staining. Mutants were tested for their ability to adhere to AGS cells and compared with the wild-type SS1 strain. Mutations in genes in the *cag* pathogenicity island showed that *cagP* and *cagE* mutants adhered less than the wild-type strain to AGS cells, whereas a *cagF* mutant showed no reduction in adherence. Mutations in genes involved in flagellar biosynthesis showed that the adherence ability of *fliQ*, *fliM* and *fliS* mutants was reduced, but a *flhB* mutant possessed wild-type levels of adherence. Mutations in genes coding for the urease (*ureB*) and phospholipase (*pldA*) enzymes did not affect adherence, but mutation of the *tlyA* gene encoding an *H. pylori* haemolysin resulted in a reduced adherence. A *fliQ* mutant, with reduced adherence to AGS cells, was less able to induce AGS cell apoptosis than SS1. The ability to induce G₀G₁ cell cycle arrest was also abolished in the *fliQ* mutant. However, an increased cell number in S phase was observed when AGS cells were exposed to the *fliQ* mutant compared with SS1, suggesting that unattached bacteria may still be able to stimulate cell proliferation. In addition to known adhesins, other bacterial virulence factors such as CagE, CagP, FliQ, FliM, FliS and TlyA appear to play a role in *H. pylori* adherence to gastric epithelial cells. Mutations in these genes may affect *H. pylori* pathogenicity by reducing either the ability of the bacteria to attach to gastric epithelial cells or the intensity of bacteria–host cell interactions.

Introduction

Chronic infection with *Helicobacter pylori*, the main cause of chronic gastritis, results in various disease outcomes including peptic ulceration, gastric adenocarcinoma and gastric lymphoma [1]. The bacterium colonises the gastric mucosa by adhering to and penetrating the mucus layer lining the gastric epi-

thelium [2]. Colonisation of the gastric mucous layer protects the bacteria from the extreme acidity of the gastric lumen and displacement from the stomach by forces such as those generated by peristalsis and gastric emptying. The majority of the colonising bacteria remain in the gastric mucous layer, but some adhere to the gastric epithelial cells. The clinical significance of the host–pathogen interactions that follow attachment of *H. pylori* to human gastric cells remains to be fully elucidated. It is considered unlikely that chronic infection with *H. pylori* could occur in the absence of adhesin–host cell interactions [3]. Therefore, adherence of the bacteria to the gastric mucosa is

Received 31 July 2001; revised version received 3 Dec. 2001; accepted 7 Dec. 2001.

Corresponding author: Dr Z.-W. Zhang (e-mail: z.w.zhang@bristol.ac.uk).

one of the initial steps of *H. pylori* infection and is an important virulence factor.

Bacterial surface structures play an important role in determining the mode of interaction of individual bacteria with other bacteria and with other factors in their immediate surroundings. *H. pylori* has evolved a repertoire of interactive surface molecules that enable the bacteria to adhere to human gastric epithelial cells and phagocytic cells *via* adhesins interacting with host-cell receptors. Many different *H. pylori* adhesins have been identified [4], implying that adherence is a multifactorial process. However, there is no consensus as to which adhesins are most important *in vivo*, or whether adhesin(s) and receptor(s) for the *H. pylori*–epithelial cell interactions are similar to the *H. pylori*–phagocytic cell interactions. Furthermore, although adhesins are a group of bacterial surface molecules, their adhesive functions may be inhibited or enhanced by other bacterial virulence factors during the processes of adhesin synthesis, transportation, secretion and activation. The real challenge in this post-genomic era is not only to study known adhesins to determine which are important *in vivo*, but also to identify other bacterial factors which may have an effect on *H. pylori* adherence.

At least five different *H. pylori* adhesins have been described [4]. One group of putative adhesins is the *H. pylori* outer-membrane protein (Hop) family, represented in the strain 26695 genome sequence by 32 members [5]. Three previously identified *H. pylori* adhesins belong to the Hop family [6–8]: AlpA and AlpB, which may act as adhesins and recognise different receptors on the gastric epithelial cell surface, and the BabA adhesin, which mediates attachment to the blood group antigen Lewis^b (Le^b) [9]. It is suggested that other members of the Hop family may also act as adhesins. Recently, Namavar *et al.* [10] identified a 16-kDa surface protein that adhered to oligosaccharide ligands such as sulphated Lewis^a (Le^a) antigens present on mucin glycoproteins. Tomb *et al.* [5] showed that the Hop genes have highly homologous domains at the 5' and 3' ends. This feature suggests the

possibility of recombination and hence an increase in antigenic variation. One of these genes, *hopZ*, has been characterised recently. A *hopZ* mutant showed significantly reduced binding to human gastric epithelial cells compared with the wild-type strain ATCC 43504 [11].

This study assessed the effect of mutations in 16 *H. pylori* virulence genes not previously associated as adhesins on bacterial adherence to gastric AGS cells. All the genes studied can be defined as virulence determinants, either through previous *H. pylori* studies or by their similarity to virulence genes in other bacteria. To further assess the importance of bacterial adherence on *H. pylori* pathogenicity, the effect of some of these mutants on AGS cell cycle progression was also investigated.

Materials and methods

Construction of defined isogenic *H. pylori* mutants

H. pylori wild-type strain SS1 was used in this study [12]. Defined isogenic *H. pylori* SS1 mutants were constructed as described previously [13]. Briefly, specific amplifying primers for each gene were designed from the *H. pylori* 26695 genome sequence. The resulting PCR products were amplified from *H. pylori* 26695 chromosomal DNA and cloned into pUC19. A defined deletion and unique *Bgl*II site were introduced into each of the cloned genes by inverse PCR mutagenesis [14, 15]. A kanamycin resistance gene was cloned into the unique *Bgl*II site and these constructs were introduced into the SS1 wild-type strain by electroporation [16]. Double-crossover mutants were selected and screened as described previously [13]. The *H. pylori* wild-type strain and the mutant strains used are listed in Table 1.

Cell culture and growth of *H. pylori* strains

Gastric AGS cells, derived from human gastric carcinomas, were grown in culture medium consisting

Table 1. *H. pylori* SS1 and its isogenic mutants

Code no.	Gene	HP no.	Function
SS1	Wild-type	...	...
ND1	<i>cagP</i>	0536	<i>cag</i> pathogenicity island; function unknown
ND4	<i>cagE</i>	0544	<i>cag</i> pathogenicity island; IL-8 induction
ND8	<i>cagF</i>	0543	<i>cag</i> pathogenicity island; function unknown
ND3	<i>ureB</i>	0072	Urease β -subunit (urea amidohydrolase)
ND5	<i>pldA</i>	0499	Phospholipase [13]
ND6	<i>flil</i>	1420	Flagellar export protein (ATP synthase) [17, 18]
SF1	<i>fliG</i>	0352	Flagellar motor switch protein [19]
SF3	<i>fliQ</i>	1419	Flagellar biosynthetic protein [19, 20]
SF4	<i>fliM</i>	1031	Flagellar motor switch protein
SF5	<i>fliH</i>	1575	Flagellar biosynthetic protein [19, 20]
MW1	<i>fliS</i>	0753	Flagellar biosynthetic protein [19]
MW2	<i>fliF</i>	0351	Flagellar basal-body M-ring protein [19]
EA2	<i>feoB</i>	0687	Iron (II) transport protein, ferrous binding protein [21]
RS1	<i>cheY1</i>	1067	Chemotaxis response regulator [22]
SF6	<i>cheAY2</i>	0392	Chemotaxis histidine kinase [22]
RS7	<i>tlyA</i>	1086	Haemolysin [23]

of RPMI 1640 medium, fetal calf serum (FCS; Sigma-Aldrich, Poole) 10%, penicillin 100 IU/ml, streptomycin 100 µg/ml and 2 mM L-glutamine. The cells were maintained in a humidified atmosphere of CO₂ 5% and air 95% at 37°C. All the reagents and medium were purchased from GibcoBRL (Paisley).

H. pylori strains were stored at -80°C in Brain Heart Infusion (BHI) broth (Oxoid) containing glycerol 15% v/v and FCS 10% v/v. Strains were grown in BHI broth supplemented with FCS 10% v/v or on *Helicobacter* selective agar (DENT), consisting of Blood Agar Base No. 2 (Oxoid) supplemented with lysed defibrinated horse blood (TCS Microbiology, Botolph Claydon) 7% v/v and DENT selective supplement (Oxoid), in a micro-aerobic atmosphere at 37°C. For the adherence assay, overnight broth cultures were resuspended in phosphate-buffered saline (PBS) to a final concentration of 5×10^8 cfu/ml ($OD_{450} \approx 1.0$). For the cell cycle analysis, the bacteria were grown on Columbia Blood Agar containing horse blood 5% (Oxoid) for 48 h under micro-aerobic conditions.

H. pylori adherence assay

The adherence assay was performed as described previously [24]. Gastric AGS cells (5×10^6 cells) and *H. pylori* (5×10^8 cfu) were incubated at 37°C for 1 h with agitation (150 rpm). Non-adherent bacteria were removed by centrifugation with 10 ml of sucrose 15% w/v solution. Cells were washed once with PBS and then incubated with a 1 in 5 dilution of polyclonal anti-*H. pylori* antibody (SkyTek Laboratories, Logan, USA) on ice for 30 min. After washing with 15 ml of PBS, the cells were incubated for an additional 30 min on ice in a 1 in 20 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma-Aldrich). The cells were washed and resuspended in 1 ml of formaldehyde 1%. Before flow cytometric analysis, the adherence of the bacteria to AGS cells was visualised by fluorescence microscopy. A FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) was used to measure bacteria adhering to AGS cells. By using a dot-plot display of forward light scatter and side (90°) light scatter, the machine was gated to include single cells and to exclude cell debris and unbound bacteria. Fluorescence data were acquired in log-model on a 256-channel scale by an analysis of 10 000 individual cells. The results were expressed as the positive percentage of fluorescent cells calculated from fluorescence frequency distribution histograms. The threshold of cells without adherent bacteria was established for each experiment, the cells being stained with the two-step FITC-conjugated *H. pylori* antibody method described above. All experiments were repeated at least three times.

Flow cytometric analysis for gastric epithelial apoptosis and cell cycle phase distribution

Subconfluent AGS cells were incubated with or without *H. pylori* (bacteria:cell ratio 100:1) for 48 h. The effect of *H. pylori* on gastric epithelial cell cycle phase distribution was assessed by flow cytometry as described previously [25]. Briefly, a pool of detached and adherent cells was washed in PBS, then the cells were fixed in ice-cold ethanol 70% overnight. The cell pellets were resuspended in 1 ml of a solution containing ribonuclease (Sigma-Aldrich) 200 µg/ml and propidium iodide (Sigma-Aldrich) 50 µg/ml for 60 min at 37°C. Cell cycle analysis was performed on a FACScan flow cytometer equipped with FACStationTM and CellQuest software (Becton Dickinson Immunocytometry Systems, Oxford). Debris was eliminated from the analysis with a forward angle light scatter threshold. Cell doublets and clumps were gated out of the analysis with a dot-plot display of area and width; 10 000 cells were analysed for each sample and the apoptotic cells were considered to constitute the sub-G₁ cell population. All experiments were repeated at least three times.

Statistical analysis

Any significance in differences between two data sets was determined by the Student's *t* test and by single-factor ANOVA for more than two data sets. *p* values <0.05 were considered significant in all analyses.

Results

Effect of H. pylori strains on bacterial adherence to gastric AGS cells

The morphology and growth rate of the 16 isogenic SS1 mutants were assessed and showed no significant differences when compared to the wild-type strain. The ability of these mutants to adhere to cultured human AGS cells was analysed (Table 2). The results are presented as a percentage figure calculated from the number of AGS cells adhered to by *H. pylori* strains and the total number of AGS cells analysed by flow cytometry. Statistical analysis was performed on each data set and a reduction in the ability of an individual mutant to adhere to AGS cells was deemed significant if *p* <0.05. Reduced adherence was observed with mutations of the *cagP*, *cagE*, *fliQ*, *fliM*, *fliS* and *tlyA* genes. The reduction in adherence to AGS cells is obviously not a result of the introduction of the kanamycin resistance cassette during mutagenesis, as many *H. pylori* SS1 mutants showed wild-type levels of adherence.

Effect of H. pylori strains on gastric AGS cell apoptosis and cell cycle progression

To examine the effect of *H. pylori* adherence on the pathogenicity of the bacterium, gastric AGS cell cycle

Table 2. Bacterial surface structures and *H. pylori* adherence to gastric AGS cells

Code no.	Gene	Mean (SD) adherence	p value
SS1	Wild-type	92.8 (2.6)	...
ND1	<i>cagP</i>	75.7 (17.7)	0.01
ND4	<i>cagE</i>	52.1 (9.7)	<0.0001
ND8	<i>cagF</i>	92.3 (7.8)	0.9
ND3	<i>ureB</i>	95.6 (2.6)	0.3
ND5	<i>pldA</i>	92.0 (1.3)	0.1
ND6	<i>fliI</i>	99.3 (0.5)	0.1
SF1	<i>fliG</i>	96.9 (4)	0.38
SF3	<i>fliQ</i>	62.8 (13.2)	0.0001
SF4	<i>fliM</i>	58.8 (5.6)	<0.0001
SF5	<i>flhB</i>	96.8 (3.7)	0.1
MW1	<i>fliS</i>	58 (5.3)	<0.0001
MW2	<i>fliF</i>	96.6 (1.2)	0.3
EA2	<i>feoB</i>	91.6 (4.5)	0.8
RS1	<i>cheY1</i>	83.5 (15.1)	0.1
SF6	<i>cheAY2</i>	95.1 (6.4)	0.4
RS7	<i>tlyA</i>	71.4 (7.5)	<0.0001

distribution was further analysed after exposure to the *H. pylori* SS1 wild-type strain and *ureB*, *pldA* and *fliQ* isogenic mutants. A significant induction of AGS cell apoptosis was observed by incubating either with the wild-type strain or its mutants ($p = 0.002$, 0.0003 , 0.0007 and 0.00001 , respectively), but the rates induced by the mutants were markedly lower than their parent strain SS1 ($p = 0.007$, 0.01 and 0.01 , respectively) (Fig. 1). There were no significant differences in the induction of AGS cell apoptosis among the three mutants. Analysis of cell cycle phase distribution of surviving cells indicated that cells exposed to strain SS1 showed significant inhibition of cell cycle progression with $62.8 \pm 1\%$ of live cells arrested in G_0G_1 phases (Fig. 2). However, the ability to induce G_0G_1 cell cycle arrest was abolished in all three mutants ($p = 0.0008$, 0.0004 and 0.001 , respectively). Interestingly, the *fliQ* mutant, which has a

Fig. 1. *H. pylori*-induced gastric AGS cell apoptosis. Sub-confluent AGS cells were incubated in the presence or absence of *H. pylori* for 48 h. The induction of AGS cell apoptosis was assessed by flow cytometry with propidium iodide staining. The results are expressed as percentage of total cells analysed and represent an average of three or more experiments. NT, untreated control. * $p < 0.01$.

reduced adherence to gastric cells, showed a marked increase in cell numbers in S phase compared with untreated control cells ($p < 0.0001$) and the wild-type strain ($p < 0.001$). The *ureB* and *pldA* mutants showed no obvious effect on the cell cycle phase distribution of surviving AGS cells when compared to untreated control cells ($p = 0.2$ and 0.1 , respectively) and to the wild-type strain ($p = 0.2$ and 0.1 , respectively).

Discussion

Clinical isolates of *H. pylori* are known to differ in virulence and those from individuals with peptic ulcers are mostly type I strains which express both VacA and CagA [26]. The *cagA* gene is a marker for a large 40-kb locus containing >40 genes, termed the *cag* pathogenicity island (*cag* PAI), and the majority of these genes encode membrane-associated proteins with features similar to other bacterial secretion systems, particularly the type IV system epitomised by *Bordetella pertussis* toxin secretion [27–29]. A *cagE* mutant induced only mild inflammation in Mongolian gerbils, whereas the wild-type strain and *vacA* mutants induced more severe gastritis [30]. Mutation of *cagE* abolishes the ability of *H. pylori* to induce the cytokine interleukin-8 (IL-8), a neutrophil chemotactic factor, in Kato-3 cells [27, 29, 31, 32]. Adherence has been shown to play a role in the induction of *H. pylori*-associated IL-8 secretion [33]. The present study has clearly shown that mutation of *cagE* markedly reduced the adherence of *H. pylori* to gastric AGS cells. This finding suggests that the inability of a *cagE* mutant to stimulate IL-8 induction may be partly due to the loss of adherence capacity to gastric epithelial cells. A recent study has also found that a *cagE* mutant failed to colonise both C57BL/6j and BALB/c mice [34]. The present study also found that a *cagP* mutant had reduced adherence to gastric AGS cells, but not a *cagF* mutant. The function of CagP and CagF has yet to be defined. Although the reasons leading to the reduced adherence capacity in these mutants remain unclear, data from the present study suggest that some genes in the *cag* PAI play a role in *H. pylori* adherence to gastric epithelial cells.

H. pylori motility is essential for colonisation [35]. Motility enables the bacterium to spread into the viscous mucous layer covering the gastric epithelium. At least 40 proteins in the *H. pylori* 26695 genome appear to be involved in the regulation, secretion and assembly of the flagellar architecture [5]. In *Salmonella enterica* serovar Typhimurium, the first structure in flagellar assembly is the MS ring (FliF). The next structure assembled is the C ring which contains the switch proteins, FliG, FliM and FliN. This is followed by rod assembly for which several proteins, including FliI, FliQ and FlhB, are required in addition to the rod structural proteins [36, 37]. These proteins, believed to be located at the cytoplasmic side of the basal body

Fig. 2. Effect of *H. pylori*-associated gastric AGS cell cycle distribution (■, G₀/G₁; □, S; ▨, G₂/M). Subconfluent AGS cells were incubated in the presence or absence of *H. pylori* for 48 h. The effect of *H. pylori* on AGS cell cycle phase distribution was assessed by flow cytometry with propidium iodide staining. The results are expressed as percentage of total cells analysed and represent an average of three or more experiments. NT, untreated control. **p* < 0.001.

near to the switch [37], are thought to be components of the flagellum-specific export apparatus, exhibiting pronounced amino acid similarities with proteins involved in export of virulence factors [36]. FliS, required for efficient elongation of the filament in *S. Typhimurium*, is thought to be a cytoplasmic chaperone for flagellin export [37]. Factors responsible for colonisation may not affect the direct adherence to the cells, as adhesion to and colonisation of the gastric mucosa are separate stages of *H. pylori* infection [19, 20]. This is supported by data from the present study which show that some of the non-motile mutants are still able to adhere to AGS cells. However, the *fliQ*, *fliM* and *fliS* mutants all showed a reduced adherence to gastric epithelial cells. This may be due to the fact that these mutants are defective in some adherence pathway. For example, FliQ is probably involved in a type III export pathway in *H. pylori* [19, 20]. Therefore, the reduced level of adherence may underline the inability of the *fliQ* mutant to export a protein with a role in adherence. In contrast, mutants such as *fliB* or *fliI*, which do not appear to be involved in the type III export pathway, showed wild-type levels of adherence. Mutations in genes involved in the regulation of chemotaxis (*cheAY2* and *cheY1*) did not appear to affect adherence to gastric epithelial cells.

Like other pathogens, *H. pylori* possesses iron-scavenging systems for survival under the low iron conditions encountered in the human host [4]. Genomic analysis suggests that *H. pylori* has several systems for iron uptake [5]. One system for iron uptake present in *H. pylori* appears to be similar to the ferrous (*feo*) iron uptake system present in *Escherichia coli*. The *feoAB* operon encodes proteins which contribute significantly

to the iron supply of *E. coli* cells under anaerobic conditions [38]. An *feoB*-like gene (HP0687), coding for a putative cytoplasmic protein with homology to ATPase, was identified in *H. pylori*, whereas no *feoA* orthologue seems to be present. Although FeoB has been shown to be important in both Fe²⁺ and Fe³⁺ transport [21, 39], the data from the present study did not support a role for this protein in *H. pylori* adherence.

One of the notable features of *H. pylori* grown on unlysed blood agar plates is haemolytic activity and it has been suggested that haemolysins may affect the biological activity of bacterial adhesins [40, 41]. It is known that *E. coli* haemolysins, by interacting with bacterial adhesins, can accelerate and enhance the rate of phagocytosis of bacteria by neutrophils and also increase the release of histamine and leukotriene from inflammatory cells [42]. Mutation of the gene encoding the *H. pylori* haemolysin TlyA significantly reduced the adherence of the organism to AGS cells. This suggests that there may be some interaction between TlyA and *H. pylori* adhesins, although the underlying mechanisms remain unclear. In contrast, mutation of the gene encoding the *H. pylori* phospholipase PldA, which also results in reduced haemolytic activity [13], showed a similar level of adherence to the wild-type strain. This suggests that PldA, whilst possessing haemolytic activity, has no role in adherence.

Bacterial adherence to host cells has been shown to be an important factor in the induction of cell cycle changes [43]. To examine whether the changes observed in *H. pylori* adherence to gastric epithelial cells have any effect on bacteria-associated cell damage, the

present study assessed *H. pylori*-induced gastric epithelial cell cycle changes after exposure to SS1 and three mutants: *ureB*, *pldA* and *fliQ*. Both UreB and PldA are important virulence factors. Mutations in these genes produced *ureB* and *pldA* mutants with normal levels of adherence to AGS cells, whereas a *fliQ* mutant showed a markedly decreased adherence ability to AGS cells. Apoptosis is a prominent feature of *H. pylori*-induced epithelial cell damage. Many factors are responsible for the induction of *H. pylori*-associated gastric epithelial cell apoptosis [44]. The data from the present study further confirm that phospholipase and urease activities plus bacterial adherence to host cells are important factors in the induction of apoptosis.

H. pylori possesses several different phospholipase (PL) activities, such as PLA₁, PLA₂ and PLC [45]. The main activities of these PLs are thought to be the degradation of the phospholipid components of the mucosal barrier and normal phospholipid bilayer of the epithelial cell membrane [46]; but it may also influence the synthesis of prostaglandin [47]. Phospholipids constitute c. 20% of the dry weight of the human gastric mucus and are believed to be important in generating a hydrophobic barrier and maintaining normal cell functions. It has been reported that mucosal hydrophobicity is reduced in *H. pylori*-positive patients with duodenal ulcer compared with those having gastritis only [48]. The significantly diminished induction of apoptosis and cell cycle arrest by the *pldA* mutant suggests that *H. pylori* phospholipase activity may play an important role in *H. pylori*-associated gastric epithelial cell damage.

H. pylori produces high levels of urease, which makes up about 6% of the total bacterial protein [49]. This enzyme breaks down urea into ammonia and carbon dioxide, providing an acid-neutralising cloud of ammonia that could protect the bacterium from gastric acidity [50]. It has become increasingly clear that this is not the only function of urease in the physiology of *H. pylori* [51]. Although previous studies have suggested that the urease activity of *H. pylori* may be important in bacterial colonisation [4, 13, 52, 53], this study did not find a role for UreB in adherence to gastric epithelial cells. This may be because factors responsible for colonisation may not affect the direct adherence to the cells as adhesion to and colonisation of the gastric mucosa are separate stages of *H. pylori* infection [37]. However, a *ureB* mutant showed a reduced ability in inducing AGS cell apoptosis and an inhibited induction of G₀G₁ cell cycle arrest. These data further characterise the role of urease activity in *H. pylori* pathogenesis. Significant reductions in the levels of apoptosis and AGS cell cycle arrest were observed after exposure to the *fliQ* mutant, which has a significantly decreased adherence to AGS cells compared with the wild-type strain. Because the *fliQ* mutant still possesses intact *H. pylori* virulence factors,

such as UreB and PldA, the reduced abilities to induce apoptosis and cell cycle arrest further demonstrate the importance of *H. pylori* adherence. Similarly, the ability of *H. pylori* to induce G₀G₁ cell cycle arrest was abolished in all three mutants. This further suggests that bacterial virulence factors and bacterial adherence are both important determinants in *H. pylori*-induced cell cycle changes. Also, cells co-incubated with the *fliQ* mutant showed a significantly increased cell number in the S phase of cell cycle compared with cells exposed to the wild-type strain and untreated control cells, suggesting that exposure to the *fliQ* mutant may stimulate AGS cell proliferation. This may represent the effect of unattached bacteria on gastric epithelial cells. Although a proportion of *H. pylori* adhere to the gastric epithelial cells of gastric tissue, the majority of the organisms in infected patients remain in the mucus layer [54]. Therefore, investigation of the behaviour of unattached bacteria in the stomach may be of clinical importance. The enhanced AGS cell number in S phase following exposure to the *fliQ* mutant suggests that *H. pylori* may stimulate gastric epithelial cell proliferation, even without adherence to the epithelial cells. Further work is required to substantiate this hypothesis.

In summary, this study assessed the adherence of 16 SS1 isogenic mutants to gastric epithelial cells by fluorescence microscopy and flow cytometry. Mutations in genes in the *cag* PAI showed that *cagP* and *cagE* had significantly reduced adhesion, whereas a *cagF* mutant showed no difference compared to SS1. Mutation of *fliQ*, *fliM* or *fliS* genes significantly reduced the adherence of *H. pylori* to AGS cells compared with the wild-type strain. Mutations of the *pldA*, *ureB* and *feoB* genes did not affect adherence, but a *tlyA* mutant showed significantly reduced adherence to AGS cells. Induction of AGS cell apoptosis was markedly reduced in cells exposed to the *pldA*, *ureB* and *fliQ* mutants. Analysis of cell cycle phase distribution of surviving cells showed that mutations in *fliQ*, *ureB* or *pldA* reduced the ability of the bacterium to induce AGS cell cycle arrest. Apart from adhesins, many other bacterial virulence factors such as CagE, CagP, FliQ, FliM, FliS and TlyA appear to play a role in *H. pylori* adherence to gastric epithelial cells. Mutation of the encoding genes may not only influence bacterial adherence, but may also have an effect on the pathogenicity of the organism.

References

1. Williams MP, Pounder RE. *Helicobacter pylori*: from the benign to the malignant. *Am J Gastroenterol* 1999; **94**(II Suppl): S11–S16.
2. Blaser MJ, Kirschner D. Dynamics of *Helicobacter pylori* colonization in relation to the host response. *Proc Natl Acad Sci USA* 1999; **96**: 8359–8364.
3. Evans DJ, Evans DG. *Helicobacter pylori* adhesins: review and perspectives. *Helicobacter* 2000; **5**: 183–195.
4. Labigne A, de Reuse H. Determinants of *Helicobacter pylori*

- pathogenicity. *Infect Agents Dis* 1996; **5**: 191–202.
5. Tomb J-F, White O, Kerlavage AR *et al.* The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 1997; **388**: 539–547.
 6. Borén T, Falk P, Roth KA, Larson G, Normark S. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. *Science* 1993; **262**: 1892–1895.
 7. Odenbreit S, Till M, Hofreuter D, Faller G, Haas R. Genetic and functional characterization of the *alpAB* gene locus essential for the adhesion of *Helicobacter pylori* to human gastric tissue. *Mol Microbiol* 1999; **31**: 1537–1548.
 8. Odenbreit S, Till M, Haas R. Optimized BlaM-transposon shuttle mutagenesis of *Helicobacter pylori* allows the identification of novel genetic loci involved in bacterial virulence. *Mol Microbiol* 1996; **20**: 361–373.
 9. Ilver D, Arnqvist A, Ögren J *et al.* *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science* 1998; **279**: 373–377.
 10. Namavar F, Sparrius M, Veerman ECI, Appelmek BJ, Vandenbroucke-Grauls CMJE. Neutrophil-activating protein mediates adhesion of *Helicobacter pylori* to sulfated carbohydrates on high-molecular-weight salivary mucin. *Infect Immun* 1998; **66**: 444–447.
 11. Peck B, Ortkamp M, Diehl KD, Hundt E, Knapp B. Conservation, localization and expression of HopZ, a protein involved in adhesion of *Helicobacter pylori*. *Nucleic Acid Res* 1999; **27**: 3325–3333.
 12. Lee A, O'Rourke J, Corazon De Ungria M, Robertson B, Daskalopoulos G, Dixon MF. A standardized mouse model of *Helicobacter pylori* infection: introducing the Sydney strain. *Gastroenterology* 1997; **112**: 1386–1397.
 13. Dorrell N, Martino MC, Stabler RA *et al.* Characterization of *Helicobacter pylori* PldA, a phospholipase with a role in colonization of the gastric mucosa. *Gastroenterology* 1999; **117**: 1098–1104.
 14. Dorrell N, Gyselman VG, Foynes S, Li SR, Wren BW. Improved efficiency of inverse PCR mutagenesis. *Biotechniques* 1996; **21**: 604, 606, 608.
 15. Wren BW, Henderson J, Ketley JM. A PCR-based strategy for the rapid construction of defined bacterial deletion mutants. *Biotechniques* 1994; **16**: 994–996.
 16. Segal ED, Tompkins LS. Transformation of *Helicobacter pylori* by electroporation. *Biotechniques* 1993; **14**: 225–226.
 17. Dunn BE, Altmann M, Campbell GP. Adherence of *Helicobacter pylori* to gastric carcinoma cells: analysis by flow cytometry. *Rev Infect Dis* 1991; **13** Suppl 8: S657–S664.
 18. Ormerod MG. Analysis of DNA – general methods. In: Ormerod MG (ed) *Flow cytometry: a practical approach*, 2nd edn. Oxford, Oxford University Press. 1994: 119–135.
 19. Jenks PJ, Foynes S, Ward SJ, Constantinidou C, Penn CW, Wren BW. A flagellar-specific ATPase (FliI) is necessary for flagellar export in *Helicobacter pylori*. *FEMS Microbiol Lett* 1997; **152**: 205–211.
 20. Porwollik S, Noonan B, O'Toole PW. Molecular characterization of a flagellar export locus of *Helicobacter pylori*. *Infect Immun* 1999; **67**: 2060–2070.
 21. Allan E, Dorrell N, Foynes S, Anyim M, Wren BW. Mutational analysis of genes encoding the early flagellar components of *Helicobacter pylori*: evidence for transcriptional regulation of flagellin A biosynthesis. *J Bacteriol* 2000; **182**: 5274–5277.
 22. Velayudhan J, Hughes NJ, McColm AA *et al.* Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. *Mol Microbiol* 2000; **37**: 274–286.
 23. Foynes S, Dorrell N, Ward SJ *et al.* *Helicobacter pylori* possesses two CheY response regulators and a histidine kinase sensor, CheA, which are essential for chemotaxis and colonization of the gastric mucosa. *Infect Immun* 2000; **68**: 2016–2023.
 24. Martino MC, Stabler RA, Zhang ZW, Farthing MJG, Wren BW, Dorrell N. The *Helicobacter pylori* pore-forming cytolysin orthologue TlyA possesses *in vitro* haemolytic activity and has a role in colonization of the gastric mucosa. *Infect Immun* 2001; **69**: 1697–1703.
 25. Xiang Z, Censini S, Bayeli PF *et al.* Analysis of expression of CagA and VacA virulence factors in 43 strains of *Helicobacter pylori* reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. *Infect Immun* 1995; **63**: 94–98.
 26. Censini S, Lange C, Xiang Z *et al.* *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci USA* 1996; **93**: 14648–14653.
 27. Naumann M, Wessler S, Bartsch C *et al.* Activation of activator protein 1 and stress response kinases in epithelial cells colonized by *Helicobacter pylori* encoding the *cag* pathogenicity island. *J Biol Chem* 1999; **274**: 31655–31662.
 28. Odenbreit S, Püls J, Sedlmaier B, Gerland E, Fischer W, Haas R. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science* 2000; **287**: 1497–1500.
 29. Ogura K, Maeda S, Nakao M *et al.* Virulence factors of *Helicobacter pylori* responsible for gastric diseases in Mongolian gerbil. *J Exp Med* 2000; **192**: 1601–1609.
 30. Tummuru MK, Sharma SA, Blaser MJ. *Helicobacter pylori* *picB*, a homologue of the *Bordetella pertussis* toxin secretion protein, is required for induction of IL-8 in gastric epithelial cells. *Mol Microbiol* 1995; **18**: 867–876.
 31. Segal ED, Lange C, Covacci A, Tompkins LS, Falkow S. Induction of host signal transduction pathways by *Helicobacter pylori*. *Proc Natl Acad Sci USA* 1997; **94**: 7595–7599.
 32. Rieder G, Hatz RA, Moran AP, Walz A, Stolte M, Enders G. Role of adherence in interleukin-8 induction in *Helicobacter pylori*-associated gastritis. *Infect Immun* 1997; **65**: 3622–3630.
 33. Fisher KJ, Kolesnikow T, Mitchell HM, Wilson JE, O'Rourke J, Lee A. *cagE* is essential for gastric colonisation in the Sydney strain mouse model. *Gut* 1999; **45** Suppl III: A24.
 34. Suerbaum S. The complex flagella of gastric *Helicobacter* species. *Trends Microbiol* 1995; **3**: 168–171.
 35. Macnab RM. Genetics and biogenesis of bacterial flagella. *Annu Rev Genet* 1992; **26**: 131–158.
 36. Minamino T, Macnab RM. Components of the *Salmonella* flagellar export apparatus and classification of export substrates. *J Bacteriol* 1999; **181**: 1388–1394.
 37. Foynes S, Dorrell N, Ward SJ *et al.* Functional analysis of the roles of FliQ and FlhB in flagellar expression in *Helicobacter pylori*. *FEMS Microbiol Lett* 1999; **174**: 33–39.
 38. Kammler M, Schön C, Hantke K. Characterization of the ferrous iron uptake system of *Escherichia coli*. *J Bacteriol* 1993; **175**: 6212–6219.
 39. van Vliet AHM, Wooldridge KG, Ketley JM. Iron-responsive gene regulation in a *Campylobacter jejuni* *fur* mutant. *J Bacteriol* 1998; **180**: 5291–5298.
 40. Segal ED, Tompkins LS. Identification and characterization of a *Helicobacter pylori* hemolysin. *Infect Agents Dis* 1993; **2**: 178–182.
 41. Drazek ES, Dubois A, Holmes RK *et al.* Cloning and characterization of hemolytic genes from *Helicobacter pylori*. *Infect Immun* 1995; **63**: 4345–4349.
 42. Scheffner J, König W, Hacker J, Goebel W. Bacterial adherence and hemolysin production from *Escherichia coli* induces histamine and leukotriene release from various cells. *Infect Immun* 1985; **50**: 271–278.
 43. Fan X, Gunasena H, Cheng Z *et al.* *Helicobacter pylori* urease binds to class II MHC on gastric epithelial cells and induces their apoptosis. *J Immunol* 2000; **165**: 1918–1924.
 44. Zhang ZW, Farthing MJG. Molecular mechanisms of *Helicobacter pylori* associated gastric carcinogenesis. *World J Gastroenterol* 1999; **5**: 369–374.
 45. Ottlecz A, Romero JJ, Hazell SL, Graham DY, Lichtenberger LM. Phospholipase activity of *Helicobacter pylori* and its inhibition by bismuth salts. Biochemical and biophysical studies. *Dig Dis Sci* 1993; **38**: 2071–2080.
 46. Nilius M, Malfetheriner P. *Helicobacter pylori* enzymes. *Aliment Pharmacol Therapeut* 1996; **10** Suppl 1: 65–71.
 47. Eberhart CE, Dubois RN. Eicosanoids and the gastrointestinal tract. *Gastroenterology* 1995; **109**: 285–301.
 48. Asante M, Ahmed H, Patel P *et al.* Gastric mucosal hydrophobicity in duodenal ulceration: role of *Helicobacter pylori* infection density and mucus lipids. *Gastroenterology* 1997; **113**: 449–454.
 49. Jones AC, Logan RPH, Foynes S, Cockayne A, Wren BW, Penn CW. A flagellar sheath protein of *Helicobacter pylori* is identical to HpaA, a putative N-acetylneuraminylactose-binding hemagglutinin, but is not an adhesin for AGS cells. *J Bacteriol* 1997; **179**: 5643–5647.
 50. Marshall BJ, Barrett LJ, Prakash C, McCallum RW, Guerrant RL. Urea protects *Helicobacter* (*Campylobacter*) *pylori* from

- the bactericidal effect of acid. *Gastroenterology* 1990; **99**: 697–702.
51. Hazell SL, Mendz GL. How *Helicobacter pylori* works: an overview of the metabolism of *Helicobacter pylori*. *Helicobacter* 1997; **2**: 1–12.
52. Mobley HLT, Hu L-T, Foxall PA. *Helicobacter pylori* urease: properties and role in pathogenesis. *Scand J Gastroenterol* 1991; **26** Suppl 187: 39–46.
53. Moran AP. Pathogenic properties of *Helicobacter pylori*. *Scand J Gastroenterol* 1996; **31** Suppl 215: 22–31.
54. Lee A, Mitchell H. Basic bacteriology of *H. pylori* colonization factors. In: Hunt RH, Tytgat GNT (eds) Basic mechanisms to clinical cure. Dordrecht, Kluwer Academic Publishers. 1994: 59–72.