INTRODUCTION

*Helicobacter pylori* strains infect the stomachs of half of the world’s population. They cause gastritis and gastric and duodenal ulceration, and are a major risk factor for the development of mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma. A number of virulence factors are associated with disease outcome, including the vacuolating cytotoxin (VacA) and the possession of the cytotoxin-associated gene pathogenicity island (*cag* PAI) comprising 27–31 genes (Censini et al., 1996).

The *cag* gene is present in virtually all strains of *H. pylori* but is polymorphic (Atherton et al., 1997), comprising variable signal regions (type s1 or s2) and mid-regions (type m1 or m2). Type s1/m1 VacA causes more epithelial cell damage than type s1/m2, whereas type s2/m2 and the rare s2/m1 are non-toxic due to the presence of a short 12-residue hydrophilic extension on the s2 form (Letley et al., 2003). VacA forms anion-selective channels within artificial membranes (Czajkowsky et al., 1999) and is assumed to do the same in vivo, increasing permeability to anions and urea (Tombola et al., 2001). Endocytosis of VacA channels leads to the formation of large vacuoles within the late endosome–lysosome compartment.

The *cag* PAI encodes a type IV secretory system that causes inflammation by activation of NF-κB and secretion of cytokines and chemokines such as interleukin 8 (IL-8) (Tummuru et al., 1995; Censini et al., 1996; Keates et al., 1997; Viala et al., 2004; Brandt et al., 2005), and facilitates the translocation of CagA into the cytosol of epithelial cells, where it becomes tyrosine phosphorylated by Src kinases (Asahi et al., 2000; Selbach et al., 2002; Stein et al., 2002). Phosphorylated CagA interacts with SHP-2 phosphatase (Higashi et al., 2002a, b; Yamazaki et al., 2003) and results in the formation of long needle-like cellular protrusions referred to as the ‘hummingbird’ phenotype (Segal et al., 1996, 1999). This phenotype is considered to be pro-proliferative, so may be important in carcinogenesis.

Functional association between the *Helicobacter pylori* virulence factors VacA and CagA

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The *Helicobacter pylori* virulence factors CagA and VacA are implicated in the development of gastroduodenal diseases. Most strains possessing CagA also possess the more virulent vacuolating form of VacA. This study assessed the significance of possession of both virulence factors in terms of their effect on gastric epithelial cells, using a set of minimally passaged, isogenic VacA, CagA and CagE mutants in *H. pylori* strains 60190 and 84-183. The *cag* A and *cagE* mutants were found to significantly increase VacA-induced vacuolation of epithelial cells, and the *vacA* mutants significantly increased CagA-induced cellular elongations, compared with wild-type strains, indicating that CagA reduces vacuolation and VacA reduces hummingbird formation. Although epithelial cells incubated with the wild-type *H. pylori* strains may display both vacuolation and hummingbird formation, it was found that (i) hummingbird length was significantly reduced in vacuolated cells compared with those without vacuolation; (ii) the number of vacuoles was significantly reduced in vacuolated cells with hummingbird formation compared with those without hummingbirds; and (iii) cells displaying extensive vacuolation did not subsequently form hummingbirds and vice versa. VacA did not affect the phosphorylation of CagA. These data show that VacA and CagA downregulate each other’s effects on epithelial cells, potentially allowing *H. pylori* interaction with cells whilst avoiding excessive cellular damage.

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Abbreviations: *cag* PAI, cytotoxin-associated gene pathogenicity island; IL-8, interleukin 8; VacA, vacuolating cytotoxin.
**METHODS**

**H. pylori strains.** *H. pylori* strains were grown on blood agar plates in a humidified microaerobic environment within a MACS-VA500 cabinet (Don Whitley Scientific). The *H. pylori* strains used were 60190 (ATCC 49503), 84-183 (ATCC 53726), 93-67 (Atherton et al., 1995) and Tx30a (ATCC 51932). Isogenic *vacA*, *cagA* and *cagE* mutants of *H. pylori* strains 60190 and 84-183 were constructed as described previously (Bebb et al., 2003; Rittig et al., 2003; Boughan et al., 2006). All isogenic mutants were re-derived and were minimally passaged along with their wild-type parental strains (maximum of six passages).

**Vacuolation assay.** AGS and MKN28 gastric epithelial cells were grown in 96-well plates until semi-confluent, before co-culture with *H. pylori* strains for 18–24 h (Argent et al., 2004a). After incubation, vacuolation was measured either by direct counting of randomly chosen microscopic fields or by use of a neutral red uptake assay (Cover et al., 1991).

**CagA translocation and phosphorylation, IL-8 ELISA and hummingbird formation.** CagA phosphorylation, following translocation from *H. pylori* into AGS cells, was carried out as described previously (Argent et al., 2004b). Briefly, AGS cells were co-cultured with *H. pylori* strains for 2, 6 or 24 h at 37 °C in an air-humidified atmosphere before the medium was saved to measure secreted IL-8. The cells were then washed, harvested and lysed in sample buffer, prior to analysis by SDS-PAGE and Western blotting with anti-CagA and anti-phosphotyrosine monoclonal antibodies. IL-8 ELISA was carried out using a DuoSet human IL-8 ELISA kit (R&D Systems). Hummingbird cell formation and measurements of hummingbird protrusions were carried out as described previously (Argent et al., 2004b).

**Statistical analysis.** Statistical analysis was performed using a two-tailed Student’s *t*-test.

**RESULTS AND DISCUSSION**

**Characterization of isogenic mutants**

*H. pylori* strains possessing toxigenic s1 forms of VacA and the *cag* PAI induce epithelial cells to produce large vacuoles in response to VacA uptake, to transform into the hummingbird phenotype following translocation and phosphorylation of CagA, and to induce secretion of IL-8. We therefore initially characterized our set of newly derived, minimally passaged, isogenic mutants in terms of these effects. VacA was not produced by the isogenic *vacA* mutant, nor did it induce AGS or MKN28 cell vacuolation (not shown). The *vacA* null mutant, however, had no effect on cytosolic delivery of CagA or its phosphorylation (Fig. 1a). CagA was not produced by the isogenic *cagA* mutant, nor was CagA translocated into AGS cells (Fig. 1a).

**Fig. 1.** Characterization of isogenic *H. pylori* mutant strains. (a) Lysates of wild-type (WT) *H. pylori* strains 60190 and 84-183 and their isogenic *cagA*, *cagE* and *vacA* mutants were subjected to SDS-PAGE and Western blotting with anti-CagA antibodies (upper two panels). *H. pylori* strains were co-cultured with AGS cells for 6 h before the cells were lysed and samples were analysed by SDS-PAGE and Western blotting with anti-phosphotyrosine antibodies (lower two panels). The isogenic *cagE* mutants did not form the type IV secretory system and therefore did not deliver CagA into the host cell cytosol. (b) IL-8 ELISA of AGS cell supernatants following co-culture with wild-type and isogenic mutant *H. pylori* strains for 6 h. IL-8 secretion was calculated as a percentage of that induced by the wild-type parental strain. IL-8 secretion for AGS cells alone and for *H. pylori* strain Tx30a was calculated relative to strain 60190. **, *p* < 0.001.
The isogenic \textit{cagE} mutant was characterized by its failure to deliver CagA into AGS cells, although the mutant had no effect on the production of CagA (Fig. 1a). The \textit{cagE} null mutant also failed to induce IL-8 secretion from AGS cells, whereas the \textit{vacA} and \textit{cagA} null mutants were unimpaired in their ability to induce secretion of this chemokine (Fig. 1b).

\textbf{CagA modulates VacA-induced vacuolation of AGS cells}

Co-culture of wild-type strains and the \textit{cagA} and \textit{cagE} mutants with AGS cells (m.o.i. 0.1–60) for 24 h resulted in vacuolation of these cells. Quantification of vacuolation using a neutral red uptake assay showed that vacuolation was significantly \((P<0.02)\) enhanced by both the \textit{cagA} and \textit{cagE} mutants (Fig. 2), indicating that CagA modulates VacA-induced vacuolation of AGS cells. In support of this, Asahi \textit{et al.} (2003) showed an increase in ‘vesicle’ formation by non-quantitative microscopy with a \textit{cagA} mutant of \textit{H. pylori} strain NCTC 11637.

\textbf{VacA modulates CagA-induced gastric epithelial cell hummingbird formation}

Having shown that CagA decreases VacA-induced vacuolation, we next looked at the effect of VacA on hummingbird formation induced by CagA, initially by co-culturing AGS cells with \textit{H. pylori} strain 60190 or the isogenic 60190 \textit{vacA} null mutant (m.o.i. ~100) for 24 h. Although AGS cells displayed the hummingbird phenotype in both cases, we found that the \textit{vacA} mutant induced significantly \((P=0.0002)\) more AGS cells to produce cell elongations (Fig. 3a) and to produce significantly \((P=0.001)\) longer cellular protrusions (Fig. 3b), suggesting that VacA downregulates hummingbird formation. This effect was more apparent in MKN28 cells, which are more susceptible to the vacuolating potential of VacA (Fig. 3c–e). MKN28 cells co-cultured with wild-type strain 60190 displayed extensive vacuolation but only a few hummingbird protrusions (Fig. 3c), comparable to that of the 60190 \textit{cagA} mutant (Fig. 3d), whereas cells co-cultured with the \textit{vacA} mutant displayed extensive hummingbird formation (Fig. 3e). When AGS cells were co-cultured with \textit{H. pylori} strain 84-183 and the 84-183 \textit{vacA} mutant, we also observed a significant \((P=0.0004)\) increase in hummingbird formation (Fig. 3b). This clearly illustrates that disruption of \textit{vacA} induces greater levels of hummingbird formation by gastric epithelial cells. We also observed that when AGS cells were co-cultured with \textit{H. pylori} strains 60190 or 84-183, the mean length of hummingbird protrusions in those cells displaying signs of VacA-induced vacuolation was significantly shorter than in cells displaying the hummingbird phenotype alone (Fig. 3f), and assessment of the number of vacuoles per AGS cell for cells with and without hummingbird formation revealed that there were significantly fewer vacuoles formed \((P<0.0001)\) in cells displaying the hummingbird phenotype, whereas the \textit{vacA} null mutants were unimpaired in their ability to induce secretion of this chemokine (Fig. 3h). Co-culture of \textit{H. pylori} strain 93-67, which possesses a functional \textit{cag} PAI but expresses an \textit{s1/m2}-type VacA, and its \textit{vacA} null mutant with AGS cells showed that there was no statistical difference in the number of cells undergoing cellular elongation (not shown), or in the length of hummingbird induced (Fig. 3h), between the wild-type and mutant strains, as strain 93-67 does not cause vacuolation of AGS cells. Thus we demonstrated that VacA can modulate CagA-induced hummingbird formation and that CagA modulates VacA-induced vacuolation. This may potentially be advantageous to \textit{H. pylori} as it allows it to use its virulence factors to interact closely with epithelial cells, but also allows down-regulation of excessive effects that could lead to epithelial damage. Indeed, a recent paper has shown that CagA-induced nuclear factor of activated T cells (NFAT) transcription was counteracted by VacA (Yokoyama \textit{et al.}, 2005). CagA activates the calcium-dependent phosphatase calcineurin via phospholipase C\textgamma{} leading to nuclear translocation of NFAT, whereas VacA pores may decrease calcium influx to prevent activation of calcineurin (Yokoyama \textit{et al.}, 2005), so it is unlikely that modulation of morphological changes caused by VacA and CagA would occur through this pathway. Nevertheless, CagA activates many signalling pathways in gastric epithelial cells, so it is possible that modulation of vacuolation may occur by an alternative mechanism and vice versa.

\textbf{VacA does not alter CagA phosphorylation within AGS cells}

We surmised that, as VacA reduces the extent and degree of hummingbird formation by gastric epithelial cells, VacA

\begin{figure}[h]
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\caption{CagA reduces VacA-induced vacuolation of AGS cells. AGS cells were co-cultured with \textit{H. pylori} strain 60190 (●) and isogenic \textit{cagA} (▲), \textit{cagE} (▲) and \textit{vacA} (×) mutants at an m.o.i. of 0.1–60 (OD$_{590}$ 0.000033–0.02) for 24 h before vacuolation was assessed by a neutral red uptake assay. *\(P<0.02\); **\(P<0.001\); ***\(P<0.0001\) for isogenic \textit{cagA} and \textit{cagE} mutants relative to strain 60190 at OD$_{590}$ 0.02–0.0008. There was no significant difference \((P>0.05)\) in neutral red uptake between the isogenic \textit{cagA} and \textit{cagE} mutants.}
\end{figure}
**Fig. 3.** VacA reduces CagA-induced gastric epithelial cell hummingbird formation. (a, b, f–i) AGS cells were co-cultured with *H. pylori* strains 60190 (a, b, f, g and i), 84-183 (b and q), 93-67 (h) and the isogenic vacA mutants of strains 60190 (a and i), 84-183 (b) and 93-67 (h) for 2 h (i), 6 h (f) or 24 h (a, b, f, g, h and i), before cells were either examined microscopically for hummingbird formation (a, b, f, g and h) and vacuolation (f and g), or lysed prior to SDS-PAGE and Western blotting (i). The blot (i) was initially probed with anti-phosphotyrosine antibodies and was then stripped and reprobed with anti-CagA antibodies. The 130 kDa phospho-CagA protein is indicated with an arrowhead. C, AGS cells only. *, P < 0.01, **, P < 0.001, ***, P < 0.0001. (c–e) MKN28 cells were co-cultured with *H. pylori* strain 60190 (c) and the isogenic cagA (d) and vacA (e) mutants for 24 h before the cells were examined microscopically.

may modulate this effect by affecting CagA phosphorylation within epithelial cells. However, we found that there was no difference in the degree of CagA phosphorylation after 2, 6 or 24 h co-culture between *H. pylori* strain 60190 and the vacA null mutant (Fig. 3i), as determined by densitometry, or between *H. pylori* strain 84-183 and the isogenic VacA mutant (not shown), indicating that VacA does not appear to modulate epithelial cell cytoskeletal rearrangements by affecting tyrosine phosphorylation of CagA.

**Hummingbird formation prevents VacA-induced vacuolation, and vacuolation prevents CagA-induced hummingbird formation**

Our work suggested that CagA and VacA downregulate each other’s effects, but did not show the level at which this occurs. We hypothesized that this may be at the level of the final phenotype, i.e. cells that have undergone CagA-induced phenotypic change would be resistant to VacA-induced vacuolation and vice versa. To test this, we co-cultured AGS cells with the cagA mutant of *H. pylori* strain 60190 or the vacA mutant of strain 93-67, or *H. pylori* strain Tx30a (s2/m2 non-vacuolating VacA, cag PAI-negative) as a control, for 1 day until the cells had vacuolated or formed hummingbirds. After removing the previous strain, we added strain 93-67 vacA mutant to the vacuolated cells, and strain 60190 cagA mutant to the AGS cells displaying the hummingbird phenotype, and incubated for a further day before cells were examined microscopically. We observed that AGS cells co-cultured with strain Tx30a became vacuolated when strain 60190 cagA mutant was added, and formed hummingbirds when strain 93-67 vacA mutant was added (not shown). However, when strain 60190 cagA mutant was added to AGS hummingbird cells, none of these cells displayed any extensive signs of vacuolation; only cells that had not formed hummingbirds became vacuolated. Similarly, when strain 93-67 vacA mutant was added to vacuolated cells, none of the vacuolated cells subsequently formed hummingbirds (not shown).

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**REFERENCES**


