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 (PAI) comprising 27–31 genes (Censini et al., 1996).

The *vacA* 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.

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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; 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).
The isogenic 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 cagE null mutant also failed to induce IL-8 secretion from AGS cells, whereas the vacA and cagA null mutants were unimpaired in their ability to induce secretion of this chemokine (Fig. 1b).

CagA modulates VacA-induced vacuolation of AGS cells

Co-culture of wild-type strains and the cagA and 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 cagA and cagE mutants (Fig. 2), indicating that CagA modulates VacA-induced vacuolation of AGS cells. In support of this, Asahi et al. (2003) showed an increase in ‘vesicle’ formation by non-quantitative microscopy with a cagA mutant of H. pylori strain NCTC 11637.

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 gastric epithelial cells. We also observed that when AGS cells were co-cultured with 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 (Fig. 3g). Co-culture of H. pylori strain 93-67, which possesses a functional cag PAI, but expresses an s1/m2-type VacA, and its 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 H. pylori as it allows it to use its virulence factors to interact closely with epithelial cells, but also allows downregulation 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) translocation was counteracted by VacA (Yokoyama et al., 2005). CagA activates the calcium-dependent phosphatase calcineurin via phospholipase Cγ leading to nuclear translocation of NFAT, whereas VacA pores may decrease calcium influx to prevent activation of calcineurin (Yokoyama 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.

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

Fig. 2. CagA reduces VacA-induced vacuolation of AGS cells. AGS cells were co-cultured with H. pylori strain 60190 (●) and isogenic cagA (●), cagE (▲) and vacA (×) mutants at an m.o.i. of 0.1–60 (OD590 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 cagA and cagE mutants relative to strain 60190 at OD590 0.02–0.0008. There was no significant difference (P > 0.05) in neutral red uptake between the isogenic cagA and cagE mutants.
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


