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

Richard H. Argent,^{1,2†} Rachael J. Thomas,^{1,2†} Darren P. Letley,^{1,2}
Michael G. Rittig,³ Kim R. Hardie¹ and John C. Atherton^{1,2}

Correspondence

Richard H. Argent
richard.argent@nottingham.ac.uk

¹Institute of Infection, Immunity and Inflammation, Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD, UK

²Wolfson Digestive Diseases Centre, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH, UK

³Centre for Biochemistry and Cell Biology, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH, UK

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 *cagA* 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.

Received 21 June 2007

Accepted 23 October 2007

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 *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- κ B 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.

†These authors contributed equally to this work.

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

H. pylori strains possessing the *cag* PAI are most likely to possess the more toxic s1 forms of VacA, whereas strains lacking the PAI generally possess non-toxic s2 forms. This is not due to genetic linkage, as the genes are distant on the *H. pylori* chromosome, nor due to clonality, as *H. pylori* is highly recombinational. The presence of s1-type *vacA*, *cagA* and the blood group-binding adhesin (*babA2*) in German strains was significantly associated with ulceration and gastric cancer (Gerhard *et al.*, 1999), suggesting that possession of multiple virulence factors increases the risk of developing gastroduodenal diseases. We hypothesized that VacA and CagA potentiate each other's effects at the level of the epithelial cell. Thus we aimed to address the hypothesis that VacA would potentiate the effects of CagA on epithelial cells and to confirm, extend and quantify the effects of CagA on VacA-induced vacuolation using an isogenic mutant approach.

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 *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 cell formation induced by CagA, initially by co-culturing AGS cells with *H. pylori* strain 60190 or the isogenic 60190 *vacA* null mutant (m.o.i. ~100) for 24 h. Although AGS cells displayed the hummingbird phenotype in both cases, we found that the *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 *cagA* mutant (Fig. 3d), whereas cells co-cultured with the *vacA* mutant displayed extensive hummingbird formation (Fig. 3e). When AGS cells were co-cultured with *H. pylori* strain 84-183 and the 84-183 *vacA* mutant, we also observed a significant ($P=0.0004$) increase in hummingbird formation (Fig. 3b). This clearly illustrates that disruption of *vacA* induces greater levels of hummingbird formation 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 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 *et al.*, 2005). CagA activates the calcium-dependent phosphatase calcineurin via phospholipase C_y 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.

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 (OD₅₅₀ 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 OD₅₅₀ 0.02–0.0008. There was no significant difference ($P > 0.05$) in neutral red uptake between the isogenic *cagA* and *cagE* mutants.

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. 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 g), 93-67 (h) and the isogenic *vacA* mutants of strains 60190 (a, b and i), 84-183 (b) and 93-67 (h) for 2 h (i), 6 h (i) 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).

ACKNOWLEDGEMENTS

This work was funded by grants from Cancer Research UK, the Medical Research Council, UK, and the digestive disorders foundation CORE, UK. J.C.A. was funded in part by a Senior Clinical Fellowship from the Medical Research Council, UK.

REFERENCES

- Argent, R. H., McGarr, C. & Atherton, J. C. (2004a). Brefeldin A enhances *Helicobacter pylori* vacuolating cytotoxin-induced vacuolation of epithelial cells. *FEMS Microbiol Lett* **237**, 163–170.
- Argent, R. H., Kidd, M., Owen, R. J., Thomas, R. J., Limb, M. C. & Atherton, J. C. (2004b). Determinants and consequences of different levels of CagA phosphorylation for clinical isolates of *Helicobacter pylori*. *Gastroenterology* **127**, 514–523.
- Asahi, M., Azuma, T., Ito, S., Ito, Y., Suto, H., Nagai, Y., Tsubokawa, M., Tohyama, Y., Maeda, S. & other authors (2000). *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. *J Exp Med* **191**, 593–602.
- Asahi, M., Tanaka, Y., Izumi, T., Ito, Y., Naiki, H., Kersulyte, D., Tsujikawa, K., Saito, M., Sada, K. & other authors (2003). *Helicobacter pylori* CagA containing ITAM-like sequences localized to lipid rafts negatively regulates VacA-induced signalling *in vivo*. *Helicobacter* **8**, 1–14.
- Atherton, J. C., Cao, P., Peek, R. M., Jr, Tummuru, M. K. R., Blaser, M. J. & Cover, T. L. (1995). Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. *J Biol Chem* **270**, 17771–17777.
- 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**, 92–99.
- Bebb, J. R., Letley, D. P., Rhead, J. L. & Atherton, J. C. (2003). *Helicobacter pylori* supernatants cause epithelial cytoskeletal disruption that is bacterial strain and epithelial cell line dependent but not toxin VacA dependent. *Infect Immun* **71**, 3623–3627.
- Boughan, P. K., Argent, R. H., Body-Malapel, M., Park, J.-H., Ewings, K. E., Bowie, A. G., Ong, S. J., Cook, S. J., Sorenson, O. E. & other authors (2006). Nucleotide-binding oligomerization domain-1 and epidermal growth factor receptor. Critical regulators of human β -defensins during *Helicobacter pylori* infection. *J Biol Chem* **281**, 11637–11648.
- Brandt, S., Kwok, T., Hartig, R., König, W. & Backert, S. (2005). NF- κ B activation and potentiation of proinflammatory responses by the *Helicobacter pylori* CagA protein. *Proc Natl Acad Sci U S A* **102**, 9300–9305.
- 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**, 14648–14653.
- Cover, T. L., Puryear, W., Perez-Perez, G. I. & Blaser, M. J. (1991). Effect of urease on HeLa cell vacuolation induced by *Helicobacter pylori* cytotoxin. *Infect Immun* **59**, 1264–1270.
- 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**, 2001–2006.

- Gerhard, M., Lehn, N., Neumayer, N., Borén, T., Rad, R., Schepp, W., Miehlik, S., Classen, M. & Prinz, C. (1999). Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin. *Proc Natl Acad Sci U S A* **96**, 12778–12783.
- Higashi, H., Tsutsumi, R., Muto, S., Sugiyama, T., Azuma, T., Asaka, M. & Hatakeyama, M. (2002a). SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. *Science* **295**, 683–686.
- Higashi, H., Tsutsumi, R., Fujita, A., Yamazaki, S., Asaka, M., Azuma, T. & Hatakeyama, M. (2002b). Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. *Proc Natl Acad Sci U S A* **99**, 14428–14433.
- Keates, S., Hitti, Y. S., Upton, M. & Kelly, C. P. (1997). *Helicobacter pylori* infection activates NF- κ B in gastric epithelial cells. *Gastroenterology* **113**, 1099–1109.
- 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*. *J Biol Chem* **278**, 26734–26741.
- 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. *Cell Microbiol* **5**, 887–899.
- Segal, E. D., Falkow, S. & Tompkins, L. S. (1996). *Helicobacter pylori* attachment to gastric cells induces cytoskeletal rearrangements and tyrosine phosphorylation of host cell proteins. *Proc Natl Acad Sci U S A* **93**, 1259–1264.
- Segal, E. D., Cha, J., Lo, J., Falkow, S. & Tompkins, L. S. (1999). Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *Proc Natl Acad Sci U S A* **96**, 14559–14564.
- 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**, 6775–6778.
- 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**, 971–980.
- 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**, 929–937.
- Tummuru, M. K. R., Sharma, S. A. & Blaser, M. J. (1995). *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* **18**, 867–876.
- Viala, J., Chaput, C., Boneca, I. G., Cardona, A., Girardin, S. E., Moran, A. P., Athman, R., Mémet, S., Huerre, M. R. & other authors (2004). Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* *cag* pathogenicity island. *Nat Immunol* **5**, 1166–1174.
- Yamazaki, S., Yamakawa, A., Ito, Y., Ohtani, M., Higashi, H., Hatakeyama, M. & Azuma, T. (2003). The CagA protein of *Helicobacter pylori* is translocated into epithelial cells and binds to SHP-2 in human gastric mucosa. *J Infect Dis* **187**, 334–337.
- Yokoyama, K., Higashi, H., Ishikawa, S., Fujii, Y., Kondo, S., Kato, H., Azuma, T., Wada, A., Hirayama, T. & other authors (2005). Functional antagonism between *Helicobacter pylori* CagA and vacuolating toxin VacA in control of the NFAT signalling pathway in gastric epithelial cells. *Proc Natl Acad Sci U S A* **102**, 9661–9666.